October 2019

CYTOCHROME P450 MONOOXYGENASE-MEDIATED LIPID METABOLISM IN OBESITY AND COLON TUMORIGENESIS

Weicang Wang

CYTOCHROME P450 MONOOXYGENASE-MEDIATED LIPID METABOLISM IN OBESITY AND COLON TUMORIGENESIS

A Dissertation Presented

by

WEICANG WANG

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

September 2019

The Department of Food Science

CYTOCHROME P450 MONOOXYGENASE-MEDIATED LIPID METABOLISM IN OBESITY AND COLON TUMORIGENESIS

A Dissertation Presented

by

WEICANG WANG

Approved as to style and content by:

_____
Guodong Zhang, Chair

_____
Hang Xiao, Member

_____
Zhenhua Liu, Member

_____
Eric A. Decker, Department Head
Department of Food Science

DEDICATION

To my great parents, wonderful wife,

All of my families and friends

ACKNOWLEDGMENTS

I would like to express my heartfelt appreciation to my advisor, Prof. Guodong Zhang, who is an outstanding researcher and an excellent advisor to me in the past five years. From Prof. Zhang, I learn how to design novel and high-impact projects, how to improve oral and writing skills, how to meet the masters in academic meetings and work with other scientists for publications. Prof. Zhang gives me valuable suggestions and guidance to help me make progress in every research projects, from curcumin and allicin projects, to TCS and lipid signaling projects. Additionally, Prof. Zhang supports me to apply for various awards and fellowships, and encourages me to collaborate with other research labs in NIH, Harvard, and UC-Davis to perform the cutting-edge science projects. With the great support from Prof. Zhang, I totally published 11 first-author papers in top journals of food and nutritional science, including *PNAS, Science Translational Medicine* and *Cancer Research*, and received 12 outstanding awards, such as *Phi Tau Sigma Founder's Award* and *Feeding Tomorrow Graduate Scholarship* during my Ph.D. study. I just could not have these outstanding achievements and become an excellent young scientist without step-by-step guidance and support from Prof. Zhang.

I would like to thank Prof. Hang Xiao, Prof. Zhenhua Liu as my committee members as well as my co-advisors during Ph.D. study. Prof. Xiao always gives me great support from my first curcumin projects to the most recent CYP project. Prof. Xiao also supports me in awards application, and provides with me the great opportunities to perform the peer-review work as academic reviewers in food science journals. Prof. Liu gives me valuable guidance in my sEH-Wnt signaling-colon inflammation project and CYP-Apc

knockout mice-colon cancer project. Prof. Liu also helps me a lot in my AOCS award application, Ph.D. dissertation proposal, and defense. All of these supports, opportunities, and guidance from Prof. Xiao and Prof. Liu are important in my past research and future career development.

I would like to thank Prof. Bruce Hammock in UC-Davis and Dr. Darryl Zeldin in NIH for their great support in my sEH-colon inflammation and CYP knockout mice-colon cancer projects and publication; I also would like to thank Prof. Decker for the great support in my research on lipid oxidation project and recommending me in the AOCS award application; thank Prof. Lili He for providing me the opportunity to present my research in Alumni weekend and nominating me in Phi Tau Sigma Scholarship; thank Prof. Lisa Minter for the great support in my TCS project and FACS analysis; thank Prof. Yeonhwa Park for the great support in my obesity projects and lipid analysis; thank Prof. Micha Peleg for the help in my teaching assistance training. Their guidance and support have been the driving force in improving my Ph.D. study and research in UMass.

I would like to thank my previous and current lab members: With Yuxin, we work together on sEH-colon inflammation, CYP colon cancer, and lipid oxidation projects; With Haixia, we work together on TCS, TCC and lipid oxidation projects; With Kathy, we work together on curcumin degradation and antimicrobial reagent projects; With Zheyuan, we work together on curcumin/allicin lymphangiogenesis projects; With Elvira, we work together on curcumin lymphangiogenesis projects; With Yoshiki, we work together on curcumin degradation projects; With Jianan, we work together on sEH-colon

inflammation projects; With Manami, we work together on lipid oxidation projects; With Guanjun, we work together on CYP inhibitors on colon cancer projects. I would also like to thank Julia, Jingyi, Xijing, Ran, Minhao, Qin and Jingwen. Thank you all so much for your great help in our research projects, my study and life.

I would like to thank all my best friends in UMass: Zipei and Ruojie, Zili and Peiyi, Quancai and Pengye, Zhiyun and Danhui, Yiren and Taoyang, Biao and Xiao, Tianxi and Bin, Yanqi and Jingning, Zhengze and Haiyan, Gumin and Panche, Weipeng, Yanhui, Fang, Xiaoqiong, Xiaomeng, Mingyue, David, Ilker, Bingjing, Fubao and Will. It is your friendship that made my graduate studies filled with love and fun.

I would like to thank all the faculties and staffs in the Department of Food Science who helped me a lot during my graduate studies at UMass. My special appreciation to Prof. Julian McClements, Prof. Lynne McLandsborough, Prof. Matthew Moore, Prof. David A. Sela, Deby, Stacy, Mary, Fran and Chia-Yu.

Last but not least, I would like to sincerely thank my beloved parents. Thank you for your great efforts to bring me up, give me countless care and love, and provide with me the best education in the past 30 years. Thank you for your huge support to my overseas study and encouraging me to pursuit my own dreams without hesitation in the past 5 years. Your son will always be your side and together with you in the future. I would also like to thank my beautiful wife-Yuxin. Thank you for your love and support in the lab, at home, during the travel, and in daily life. Thank you for letting me always have the

feeling of being loved. You are the most important person in my life and I will try my best to create a better and warm home to you.

Yours sincerely,

Weicang

May 2019

ABSTRACT

CYTOCHROME P450 MONOOXYGENASE-MEDIATED LIPID METABOLISM IN OBESITY AND COLON TUMORIGENESIS

SEPTEMBER 2019

WEICANG WANG, B.S., NORTHWEST A&F UNIVERSITY

M.S., EAST CHINA NORMAL UNIVERSITY

Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Guodong Zhang

Colon cancer is a major public health issue: it is expected to have 140,250 new cases and 50,630 deaths during 2018, making colon cancer the third most common type of cancer and the second leading cause of cancer death in the United States. Obesity is associated with enhanced colonic inflammation, which is a major risk factor of colorectal cancer. Currently, more than 35% of adults and nearly 17% of children are obese. Considering the obesity and colon cancer epidemic in the United States, there is an urgent need to identify novel therapeutic targets for obesity and colon cancer. Here, using a LC-MS/MS-based lipidomics approach and disease mouse models, we found that: 1) CYP monooxygenase and its-derived fatty acid epoxides are decreased in adipose tissues in HFD-induced obese mice; 2) CYP monooxygenase and its-derived fatty acid epoxides are upregulated in AOM/DSS-induced colon cancer; pharmacological inhibition or genetic ablation of CYP monooxygenase attenuates AOM/DSS-induced colon cancer in mice; 3) Dietary feeding of ω-3 PUFAs-rich diets increased levels of CYP-derived EDPs

in both plasma and MC38 colorectal tumor of the treated mice, systematic treatment with EDPs suppressed growth of MC38 colorectal tumor in mice. Together, these results support that previous unappreciated CYP lipid metabolism pathway plays a vital role in the development of obesity and colon cancer. Moreover, our study identifies the major enzymes and their metabolites in CYP lipid metabolism pathway as novel therapeutic targets for treating obesity and colon cancer.

Key words: Lipid Metabolism, Cytochrome P450 Monooxygenase, Obesity, Colon Tumorigenesis

TABLE OF CONTENTS

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER 1

INTRODUCTION

1.1 Background

The enzymatic metabolism of polyunsaturated fatty acids (PUFAs), such as arachidonic acid (ARA, 20:4ω-6), leads to formation of bioactive lipid metabolites (LMs), which are important lipid signaling molecules involved in regulation of many fundamental physiological and pathological processes [1-3]. There are three major pathways involved in enzymatic metabolism of PUFAs: cyclooxygenase (COX-1 and COX-2), lipoxygenase (5-LOX, and 12/15-LOX), and cytochrome P450 (CYP). The COX pathway leads to formation of prostaglandins, which are important mediators to induce inflammation and pain; and COX-2 is the therapeutic target of many anti-inflammatory drugs on the market [1]. The LOX pathway produces leukotrienes and hydroxyl fatty acids, which are predominately pro-inflammatory and play critical roles in inflammatory diseases such as asthma [1]. The CYP pathway converts PUFAs to fatty acid epoxides, which have a variety of beneficial effects such as anti-inflammatory, cardio-protective, vasodilative, and analgesic actions [2,3]. Besides ARA, other PUFAs, including linoleic acid (LA, 18:2ω-6), α-linolenic acid (α-LA, 18:3ω-3), γ-linolenic acid (γ-LA, 18:3ω-6), dihomo-γ-linolenic acid (DGLA, 20:3ω-6), eicosapentaenoic acid (EPA, 20:5ω-3), and docosahexaenoic acid (DHA, 22:6ω-3), are also efficient alternative substrates of these enzymes and are converted to the corresponding LMs with unique biological activities [3-5]. Together, this leads to formation of a large array of LMs with diverse chemical structures, many of which have potent biological activities.

Colon cancer is a major public health issue: it is expected to have 140,250 new cases and 50,630 deaths during 2018, making colon cancer the third most common type of cancer and the second leading cause of cancer death in the United States [6]. It is important to identify novel therapeutic targets of colon cancer, in order to develop effective methods for prevention and/or treatment of colon cancer. Substantial studies have shown that the metabolites, enzymes, and receptors involved in the eicosanoid signaling contribute to the carcinogenesis of colon cancer. It would be important to discover novel therapeutic targets from the eicosanoid signaling cascade, especially previously unappreciated CYP lipid metabolism pathway, in order to develop novel agents to reduce the risks of colon cancer.

Obesity is another serious public health threat over the past decades in US: currently, more than 35% of adults and nearly 17% of children are obese [7,8]. Obesity is associated with enhanced colonic inflammation [9-11], which is a major risk factor for developing colorectal cancer [12]. Indeed, obese individuals have a 30-60% greater risk of developing colorectal cancer [13,14]. Emerging research supports the notion that the eicosanoid signaling is deregulated in obesity and plays critical roles in the pathogenesis of obesity [15,16]. However, the detailed function of CYP lipid metabolism pathway in obesity and obesity-induced colonic inflammation are unknown. Considering the obesity epidemic and the potential lethal consequence of obesity-enhanced colorectal cancer, it is important to identify novel therapeutic targets in CYP lipid metabolism pathway for obesity and its-induced colonic inflammation.

1.2 Objectives

A number of studies have confirmed the importance of inflammatory mediators, particularly inflammatory cytokines and eicosanoids, in the pathogenesis of obesity and colon cancer. Previous study focus on COX and LOX pathway, however, the function of CYP lipid metabolism pathway in the obesity and colon cancer is still unknown. The objective of this thesis is to determine the contribution of CYP lipid metabolism pathway in progression of obesity and colon cancer.

To achieve our objective, we propose the following four research projects:

Research Project1: Determine the novel biomarkers or therapeutic targets from CYP lipid metabolism pathway during obesity.

In research projects 1, we will perform three key experiments: we will (1) feed the mice with HFD to induce obesity; (2) conduct a LC-MS/MS-based lipidomics analysis on adipose tissue to explore the profiles of LMs from COX, LOX and CYP metabolizing pathways; (3) perform the gene expression analysis of major metabolizing enzymes in CYP lipid metabolism pathway.

Research Project 2: Determine the novel biomarkers or therapeutic targets from CYP lipid metabolism pathway in AOM/DSS-induced colon tumorigenesis.

In research projects 3, we will perform six key experiments: we will (1) treat the mice with AOM and DSS to induce colon cancer; (2) conduct a LC-MS/MS-based lipidomics analysis on colon tissue from cancer mice to explore the profiles of LMs from COX,

LOX and CYP metabolizing pathways; (3) perform the gene expression analysis of major metabolizing enzymes in CYP lipid metabolism pathway; (4) test the effect of pharmacological inhibition of CYP on AOM/DSS-induced colon tumorigenesis; (5) test the effect of genetic knockout of CYP on AOM/DSS-induced colon tumorigenesis; (6) test the effect of CYP-derived EpOMEs on AOM/DSS-induced colon tumorigenesis.

Research Project 3: Determine the actions of ω-3 PUFAs-derived eicosanoid metabolites on colorectal cancer.

In research projects 3, we will perform four key experiments: we will (1) establish the xenograft MC38 colorectal cancer growth model in C57BL/6 mice; (2) study the effect of ω-3 PUFAs on MC38 colorectal cancer growth by feeding the mice with control ω-6 diet (ratio of ω-6-to-ω-3 PUFA is ≈ 69.3:1), ω-3 diet (ratio of ω-6-to-ω-3 PUFA is ≈ 1.26:1), or ω-3-high diet (ratio of ω-6-to-ω-3 PUFA is ≈ 0.56:1); (3) conduct a LC-MS/MS-based lipidomics analysis on plasma, tumor and colon tissues from cancer mice to explore the profiles of LMs from COX, LOX and CYP metabolizing pathways; (4) test the effect of EDPs on MC38 colorectal cancer growth in mice.

CHAPTER 2

LITERATURE REVIEW

2.1 Lipid signaling and colorectal cancer

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related deaths in the US [6], emphasizing the need for discovering novel cellular targets which are crucial in the pathogenesis of CRC. Colonic inflammation is a major risk factor for developing CRC, therefore, targeting the pathological components involved in colonic inflammation is a promising strategy to reduce the risks of CRC [17]. Eicosanoids, which are endogenous lipid signaling molecules produced from enzymatic metabolism of polyunsaturated fatty acids (PUFAs), play essential roles in inflammatory responses and were recently implicated in cancer [18,19]. The most prominent CRC-associated eicosanoids are prostaglandins, which are produced by the cyclooxygenase-2 (COX-2) enzyme that is overexpressed in most human CRC samples [19]. Genetic knockout of *Cox-2* reduces polyp formation in azoxymethane (AOM)- or *Apc* mutation-induced CRC models [20,21]. Furthermore, clinical and epidemiological studies support that pharmacological inhibitors of COX-2, such as nonsteroidal anti-inflammatory drugs (NSAIDs), are effective in reducing the risk of CRC [19], supporting the critical importance of eicosanoid signaling in CRC. However, the gastrointestinal and cardiovascular toxicities induced by the COX-2 inhibitors have limited their clinical applications [22]. Besides COX-2, the roles of other eicosanoid pathways in colonic inflammation and CRC are not well understood [23]. It is therefore important to discover novel eicosanoid signaling pathways involved in tumorigenesis.

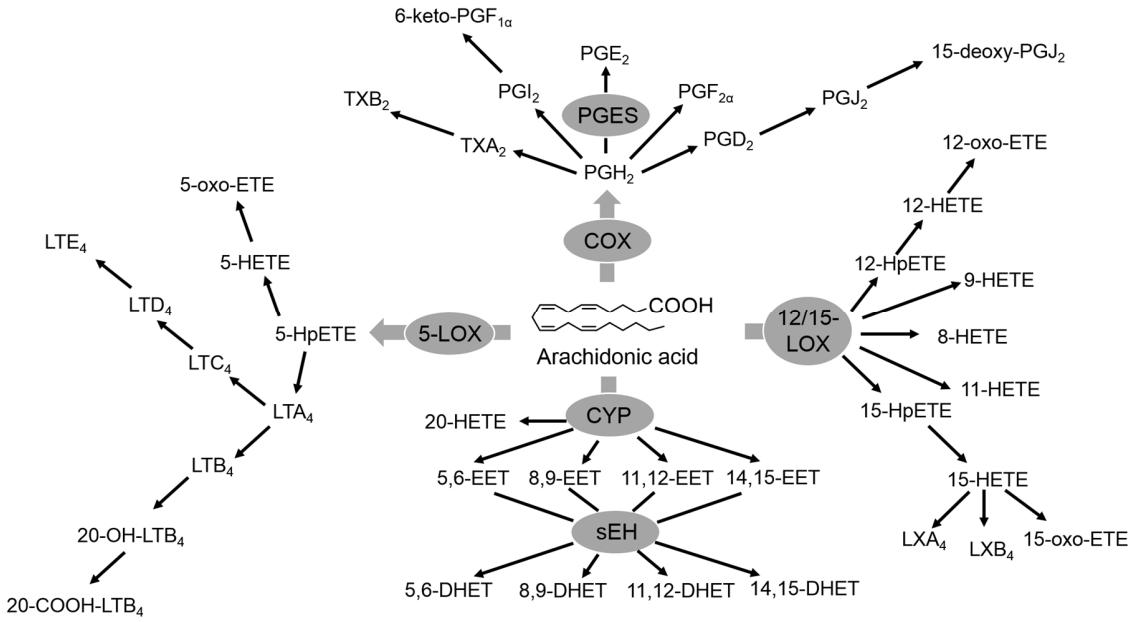

Figure 2.1 A simplified scheme of PUFA metabolism to generate bioactive lipid metabolites (LMs). There are three major metabolizing pathways: cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP).

Besides being substrates of COX-2, PUFAs are also substrates of cytochrome P450 (CYP) monooxygenases (predominately CYP2C and CYP2J isoforms), which convert them to epoxygenated fatty acids (EpFAs). EpFAs include epoxyeicosatrienoic acids (EETs) produced from arachidonic acid (ARA, 20:4ω-6), epoxyoctadecenoic acids (EpOMEs) from linoleic acid (LA, 18:2ω-6), epoxyoctadecadienoic acids (EpODEs) from α-linolenic acid (ALA, 18:3ω-3), and epoxydocosapentaenoic acids (EDPs) from docosahexaenoic acid (DHA, 22:6ω-3) [2]. EpFAs are metabolically unstable with a half-life of several seconds *in vivo*, in part because they could be rapidly metabolized by soluble epoxide hydrolase (sEH) to generate the corresponding fatty acid diols [3]. Currently the CYP eicosanoid pathway is being explored by academic laboratories and pharmaceutical companies for clinical applications. For example, GlaxoSmithKline is conducting human clinical trials to test an sEH inhibitor GSK2256294; the company has

found that this drug candidate is well-tolerated and causes sustained inhibition of sEH in humans [24]. Other novel classes of sEH inhibitors are also being considered for human trials [25]. In addition, recent studies have shown that some FDA-approved drugs are potent inhibitors of CYP monooxygenases [26].

Emerging research supports that the CYP pathway plays critical roles in regulating inflammation, angiogenesis, tumor growth, and tumor metastasis [27-30], and could be involved in the pathogenesis of colonic inflammation and CRC [31-34]. A better understanding of the roles of this previously unappreciated pathway in the pathogenesis of CRC could help to develop new strategies for cancer treatment or prevention. In this review, we will discuss recent studies of the roles of the CYP eicosanoid pathway in the pathogenesis of CRC.

2.2 Expression of CYP monooxygenases in colonic inflammation and CRC

Previous studies have shown that CYP monooxygenases are overexpressed in CRC. Enayetallah et al. reports that CYP2C9 is detected in 13 out of 17 human colon tumor samples, while it is not detected in matched benign samples [35]. Consistent with the human data, our recent study have shown that the expressions of CYP monooxygenases are increased in colon tumor tissues and colon cancer cells [34]. Compared with untreated control mice (healthy mice), the expressions of mouse *CYP monooxygenases*, such as *Cyp2c38, Cyp2c39, Cyp2c55, Cyp2c65, Cyp2c70*, *Cyp2j6, Cyp2j9,* and *Cyp2j13*, are increased in colon tissues of AOM/ dextran sulfate sodium (DSS)-treated CRC mice [34]. In addition, the concentrations of CYP-produced EpFAs are increased in the plasma and colon tumors of AOM/DSS-induced CRC mice [34]. Furthermore, we find that compared

with normal human colon cells (CCD-18co), the expression of *CYP2C8, CYP2C9, CYP2C19,* and *CYP2J2* is increased in human CRC cells (HCT116 and Caco-2) [34]. Few studies have investigated the expression of CYP monooxygenases in colonic inflammation. Willenberg et al. reported that in DSS-induced colitis models, the circulating concentrations of EpFAs are not changed [36], suggesting that the CYP eicosanoid pathway is not altered in DSS-induced colitis. Together, these results support that CYP monooxygenases are overexpressed in CRC.

In addition to CRC, previous studies have shown that CYP monooxygenases are overexpressed in other tumor tissues, such as breast, liver, and stomach tumors. There could be many mechanisms by which CYP monooxygenases are overexpressed in tumor tissues [29,35]. The expression of CYP monooxygenases has been shown to be elevated by hypoxia [3], which is a common feature of tumor tissues [37]. It is feasible that the hypoxic tumor microenvironment could contribute to the increased expression of CYP monooxygenases in tumor tissues.

2.3 Roles of CYP eicosanoid pathway in colonic inflammation and CRC

Recent studies also support the critical roles of CYP monooxygenases in tumorigenesis. In a xenograft tumor model, overexpression of human CYP2C8 or CYP2J2 in endothelial cells led to increased xenograft tumor growth of B16F10 melanoma and T241 fibrosarcoma [28]. Using a Lewis lung carcinoma (LLC) resection-induced tumor metastasis model, endothelial expression of human CYP2C8 or CYP2J2 increases lung

metastases [28]. Together, these results support the pro-tumorigenic and pro-metastatic effect of CYP monooxygenases.

Our recent studies also support a potential role of CYP monooxygenases in the tumorigenesis of CRC [34]. Compared with AOM/DSS-induced *Cyp2c*$^{+/+}$ mice, the AOM/DSS-induced *Cyp2c*$^{+/-}$ mice have lower tumor numbers and total tumor burden, as well as reduced expression of CYP monooxygenases and concentrations of CYP-derived fatty acid epoxides in colon tissues, supporting the roles of Cyp2c monooxygenases in colon tumorigenesis [34]. Consistent with the results observed in transgenic mouse models, we also find that pharmacological inhibition of CYP monooxygenases suppresses AOM/DSS-induced colon tumorigenesis in mice [34]. Together, these results support that CYP monooxygenases contribute to the tumorigenesis of CRC.

Recent research has shown that sEH plays a critical role in colonic inflammation and CRC. In DSS-induced colitis and CRC model, sEH$^{-/-}$ mice have reduced colonic inflammation (as assessed by mucosal erosion and lymphoplasmocytosis) and carcinogenesis (tumor incidence and volume) compared with wild-type mice [31]. Similarly, in an interleukin 10 (IL-10) deficiency-induced CRC model, sEH$^{-/-}$ IL-10$^{-/-}$ mice have reduced colonic expression of pro-inflammatory cytokines and formation of ulcers and carcinomas compared with sEH$^{+/+}$ IL-10$^{-/-}$ mice [32,33]. Together, these results support that sEH could contribute to colonic inflammation and inflammation-associated CRC. This is largely in agreement with previous studies which show that inhibition of sEH reduces inflammatory responses in various disease models. For example, in obese

mice, pharmacological inhibition of sEH reduces the infiltration of macrophages and expression of pro-inflammatory cytokines in epididymal fat and liver [38,39]. Deletion of sEH also leads to reduced infiltration of neutrophils, decreased levels of pro-inflammatory cytokines and less neuronal damage in an intracerebral hemorrhage mouse model [40]. Together, these studies demonstrate sEH is involved in many inflammation-associated diseases.

More studies are needed to better characterize the roles of sEH in cancer. Panigrahy et al. have shown that pharmacological inhibition or genetic deletion of sEH increases tumor growth and metastasis in xenograft models by stimulating tumor angiogenesis [28]. However, this finding is different from the results observed in the DSS and IL-$10^{-/-}$ mouse models [31-33]. The different results could be, at least in part, due to the differences in mouse models. The phenotypes in the DSS and IL-$10^{-/-}$ mouse models are strongly associated with inflammation, therefore, inhibition of sEH would reduce inflammatory response and attenuate inflammation-associated CRC [31-33]. In other models such as xenograft models, inhibition of sEH could upregulate angiogenesis and increase tumorigenesis [28]. However, we have to mention that the pro-angiogenic effects of sEH inhibition are mainly observed in mouse models [28]. A recent human clinical trial has shown that even at 100% inhibition of sEH, there is no change of the plasma concentration of vascular endothelial growth factor (VEGF), which is an important biomarker of angiogenesis [24]. Since sEH inhibitors are currently being evaluated in human clinical trials [24,25], it is of critical importance to better understand the roles of sEH and sEH inhibitors in tumorigenesis.

2.4 Effects of CYP-produced eicosanoids on colonic inflammation and CRC

The metabolism of PUFAs by CYP monooxygenases leads to the formation of EpFAs [2]. The major EpFAs in tissues and plasma include EpOMEs produced from LA, EETs from ARA, and EDPs from DHA. Emerging research supports that these eicosanoid metabolites have potent effects on tumorigenesis with the details discussed below.

Previous studies show that EpOMEs have a series of detrimental actions such as inducing chemotaxis, inflammation, cardiovascular diseases, and pulmonary injury [41-46]. In human studies, EpOMEs, which are termed "leukotoxins," are associated with multiple organ failures and adult respiratory distress syndrome in severe burn patients [42,44,47]. Notably, at a concentration as low as 10 nM, EpOMEs enhance neutrophil chemotaxis, suggesting potent pro-inflammatory effects of EpOMEs [46]. EpOMEs can be further metabolized by sEH to form the corresponding fatty acid diols termed dihydroxyoctadecenoic acids (DiHOMEs) [3]. Similar to EpOMEs, DiHOMEs have also been shown to induce chemotaxis, tissue injury, and cause mortality in animal models [47,48]. Together, these results show that metabolism of LA by CYP monooxygenases leads to the formation of pro-inflammatory eicosanoid metabolites (EpOMEs and DiHOMEs). In our recent study, we find that the administration of 12,13-EpOME exaggerates AOM/DSS-induced CRC in mice [34], supporting that EpOMEs could enhance the development of CRC.

The biological actions of EETs are more complicated, as they have been shown to have anti-inflammatory effects (negatively associated with tumorigenesis) and pro-angiogenic

effects (positively associated with tumorigenesis). Indeed, many studies have shown that EETs have potent anti-inflammatory effects. In a murine carotid artery model, treatment with 11,12-EET decreases tumor necrosis factor-α (TNF-α)–induced mononuclear cell adhesion to the arterial endothelium [49]. Treatment with 14,15-EET inhibites TNF-induced degradation of IκBα in primary human lung tissue [50] and reduces LPS-activated IL-1β and TNF-α expression in mouse macrophages [31]. Together, these results support the anti-inflammatory effects of EETs. The effects of EETs on tumor inflammation are not well characterized and require more studies. Previous studies have shown that inhibition or deletion of sEH attenuates DSS- or IL-10 knockout-induced CRC [31-33]; these results could be, at least in part, due to the anti-inflammatory effects of EETs. On the other hand, EETs have pro-angiogenic effects, and therefore could promote tumor growth and metastasis. In an orthotopic cancer model, treatment of 14,15-EET at a dose as low as 15 μg/kg/day for 28 days increases orthotopic PC3M-LN4 prostate tumor growth in SCID mice [28]. In transgenic adenocarcinoma of the mouse prostate (TRAMP) mice, administration of 14,15-EET at a dose of 15 μg/kg/day promotes prostate tumor growth, suggesting that EETs increase tumor growth *in vivo* [28]. In addition, treatment with 14,15-EET increases tumor metastasis in a LLC resection model [28]. More studies are needed to further characterize the role of EETs in tumorigenesis.

Opposite to the effects of EpOMEs and EETs, our studies have shown that ω-3 PUFA-produced EDPs inhibit angiogenesis, tumor growth, and tumor metastasis [51]. 19,20-EDP inhibits tube formation, migration, and production of matrix metalloproteinases (MMPs) in endothelial cells, and suppresses VEGF- and basic fibroblast growth factor (bFGF)-

induced angiogenesis in a Matrigel plug assay in mice, demonstrating its anti-angiogenic effect [51]. Treatment with stabilized 19,20-EDP also suppresses primary tumor growth and tumor metastasis in mice [51]. Consistent with our results, a recent study by Yanai et al. has shown that systematic treatment with EDPs inhibits pathological angiogenesis in a mouse model of macular degeneration [27]. Furthermore, our recent study has shown that treatment with EDPs suppresses growth of MC38 colon tumors, supporting the anti-CRC effects of EDPs [52].

To date, the molecular mechanisms for the biological actions of EpFAs are not well understood. Many eicosanoids act by binding to specific cellular targets. For example, COX-2-produced prostaglandin E_2 (PGE_2) and lipoxygenase (LOX)-produced leukotriene B_4 (LTB_4) act by binding to their G-protein coupled receptors [1]. The direct cellular target(s) of CYP monooxygenase metabolites are not well understood, hampering our understanding of their mechanisms of action. Previous studies show that EETs bind to cell membrane-bound proteins in a high-affinity, specific, and saturable manner [53-55], and some of the biological actions of EETs are G protein-dependent [55-58]. These results support that the CYP monooxygenase metabolites also act via interactions with specific cellular proteins; however, the identities of the cellular targets are not well understood. Beyond EETs, no study has investigated the cellular targets of other CYP monooxygenase metabolites (such as EpOMEs). Identification of their direct cellular targets could greatly enhance our understanding for the molecular mechanisms of the CYP monooxygenase pathway. In addition, the identified cellular targets could also serve as novel molecular targets for preventing or treating cancer and other human diseases.

Besides the EpFAs, their down-stream metabolites, termed fatty acid diols, have also been shown to be biologically active and could contribute to the biological actions of CYP monooxygenases and sEH. Recent studies have shown that dihydroxyeicosatrienoic acids (DHETs), which are metabolites of EETs produced by sEH, have pro-inflammatory effects [3,59]. Treatment of 5,6-DHET, 8,9-DHET, 11,12-DHET, or 14,15-DHET at a dose of 3 μM stimulates primary human monocyte migration *in vitro* [50]. Similar to the pro-inflammatory effects of ARA-produced DHETs, recent studies show the DHA-produced dihydroxydocosapentaenoic acids (DHDPs) promote progression of retinopathy in mice [60,61]. Treatment of 19,20-DHDP induces retinal angiogenesis by increasing tip cell, sprouting, and filopodia numbers in mice, all of which contributes to proliferative retinopathy [60]. Moreover, in an *ex vivo* whole mount retina model, treatment with 19,20-DHDP increases vascular endothelial cell permeability and pericyte migration into extravascular space, both of which are disease markers for diabetic retinopathy [61]. In cultured murine brain microvascular endothelial cells, treatment of 19,20-DHDP decreases junction formation between cells and reduces expression of N-cadherin, which could contribute to effect of 19,20-DHDP on vascular permeability during diabetic retinopathy [61]. Overall, these studies demonstrate that fatty acid diols are bioactive eicosanoids which could contribute to the pathogenesis of many diseases.

Together, these results support that CYP-produced eicosanoid metabolites, including EpOMEs, EETs, and EDPs, have potent effects on tumorigenesis. These results could help to establish a novel mechanistic linkage between fatty acid intake and cancer risks.

For example, animal experiments have shown that a high dietary intake of LA increases AOM-induced colon tumorigenesis, suggesting its potential adverse effect on CRC [62-66]. Here our study about the promoting effects of LA-produced EpOMEs on CRC suggests that formation of EpOMEs contribute to promoting effects of LA on the risks of CRC [34].

2.5 Roles of CYP eicosanoid pathway in obesity-induced colonic inflammation

More than one-third of US adults (34.9% or 78.6 million) are obese [67], and obese individuals have 30-60% higher risk of developing CRC [13,68]. Considering the obesity epidemic and the potential lethal consequence of CRC, obesity-enhanced CRC is a serious health problem in the US. However, the mechanism by which obesity increases the risks of CRC are not well understood, and there are few effective strategies to prevent obesity-enhanced CRC [14]. Using LC-MS/MS-based metabolomics, our recent research suggests that sEH could be a novel therapeutic target of obesity-induced colonic inflammation [69]. In a high fat diet (HFD)-induced obesity model in C57BL/6 mice, we find that the expression of sEH and the concentrations of sEH-produced fatty acid diols are significantly increased in the colon tissues of HFD-induced obese mice [69]. Furthermore, genetic ablation of sEH abolishes HFD-induced colonic inflammation in mice, with reduced expression of pro-inflammatory cytokines [69]. Furthermore, we find that the ablation of sEH attenuates HFD-induced activation of Wnt signaling pathway in colon tissues [69].

Considering the critical roles of colonic inflammation and Wnt signaling in the pathogenesis of CRC, these results support that sEH could be a potential therapeutic

target of obesity-enhanced CRC. This notion is supported by previous studies, which show that inhibition of sEH has beneficial effects on colonic inflammation, CRC, and obesity, supporting that targeting sEH is a promising strategy to reduce the risks of obesity-enhanced CRC. Previous studies have shown that: (1) compared with normal colon tissues, the expression of sEH is increased in human CRC samples [31,35]; (2) genetic ablation of sEH attenuates colonic inflammation and CRC [31-33]; and (3) sEH is overexpressed in the liver and adipose tissues of obese mice [70,71]. In addition, pharmacological inhibition or genetic ablation of sEH attenuated adverse consequences of obesity, including endoplasmic reticulum stress, metabolic syndrome, fatty liver, hepatic steatosis, inflammation, and endothelial dysfunction [16,38,39,70,72-76]. Together, these results support that targeting sEH could be a promising strategy to reduce the risks of CRC in obese individuals. Further studies are needed to characterize the roles of sEH in obesity-enhanced CRC.

In our studies, we find that the colonic expressions of CYP2C and CYP2J monooxygenases are not altered in HFD-treated mice [69]. However, considering that the fatty acid diols are down-stream metabolites of CYP monooxygenases, it is feasible that inhibition of CYP monooxygenases could also reduce obesity-induced colonic inflammation and associated tumorigenesis. Targeting CYP monooxygenases could be an alternative approach to reduce the risks of obesity-enhanced CRC. This approach could minimize potential adverse effects of sEH inhibition on angiogenesis [28], and further studies are needed to better characterize the roles of the CYP eicosanoid pathway in obesity-enhanced CRC.

CHAPTER 3

LIPIDOMIC PROFILING OF HIGH-FAT DIET-INDUCED OBESITY IN MICE: IMPORTANCE OF CYTOCHROME P450-DERIVED FATTY ACID EPOXIDES

3.1 Abstract

Enzymatic metabolism of polyunsaturated fatty acids (PUFAs) leads to formation of bioactive lipid metabolites (LMs). Previous studies have shown that obesity leads to deregulation of LMs in adipose tissues. However, most previous studies have focused on single or limited number of LMs, few systematical analyses have been carried out. We used a LC-MS/MS-based lipidomics approach, which can analyze >100 LMs produced by cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) enzymes, to analyze profile of LMs in high-fat diet-induced obesity in mice. LC-MS/MS showed that dietary feeding of high-fat diet significantly modulated profiles of LMs in adipose tissues. Among the three major PUFA metabolizing pathways (COX, LOX, and CYP), CYP-derived fatty acid epoxides were the most dramatically altered LMs. Almost all types of fatty acid epoxides were reduced by 70-90% in adipose tissues of high-fat diet-fed mice. Consistent with the reduced levels of fatty acid epoxides, the gene expressions of several CYP monooxygenases, including Cyp2j5, Cyp2j6, and Cyp2c44, were significantly reduced in adipose tissues of high-fat diet-fed mice. Our results showed that CYP-derived fatty acid epoxides are the most responsive LMs in high-fat diet-induced obesity, suggesting that these LMs could play critical roles in obesity.

3.2 Introduction

Obesity is a major health concern in the US: more than one-third of US adults (34.9% or 78.6 million) are obese [77]. Obese individuals have significantly increased risks of

developing many diseases, including cardiovascular diseases, hypertension, type II diabetes, and certain types of cancer [78]. The annual medical care cost to treat obesity and obesity-assocaited diseases is estimated to be around $190 billion in the US [79]. Therefore, it is of critical importance to better understand the mechanism by which obesity increases the risks of various human diseases, which could facilitate the development of effective therapeutic strategies.

The enzymatic metabolism of polyunsaturated fatty acids (PUFAs), such as arachidonic acid (ARA, 20:4ω-6), leads to formation of bioactive lipid metabolites (LMs), which are important lipid signaling molecules involved in regulation of many fundamental physiological and pathological processes [1-3]. There are three major pathways involved in enzymatic metabolism of PUFAs: cyclooxygenase (COX-1 and COX-2), lipoxygenase (5-LOX, and 12/15-LOX), and cytochrome P450 (CYP). The COX pathway leads to formation of prostaglandins, which are important mediators to induce inflammation and pain; and COX-2 is the therapeutic target of many anti-inflammatory drugs on the market [1]. The LOX pathway produces leukotrienes and hydroxyl fatty acids, which are predominately pro-inflammatory and play critical roles in inflammatory diseases such as asthma [1]. The CYP pathway converts PUFAs to fatty acid epoxides, which have a variety of beneficial effects such as anti-inflammatory, cardio-protective, vasodilative, and analgesic actions [2,3]. Besides ARA, other PUFAs, including linoleic acid (LA, 18:2ω-6), α-linolenic acid (α-LA, 18:3ω-3), γ-linolenic acid (γ-LA, 18:3ω-6), dihomo-γ-linolenic acid (DGLA, 20:3ω-6), eicosapentaenoic acid (EPA, 20:5ω-3), and docosahexaenoic acid (DHA, 22:6ω-3), are also efficient alternative substrates of these enzymes and are

converted to the corresponding LMs with unique biological activities [3-5]. Together, this leads to formation of a large array of LMs with diverse chemical structures, many of which have potent biological activities.

Previous research has shown that LMs play critical roles in pathology of obesity. In obese Zucker rats, there is attenuated production of prostacyclin (PGI_2), which is a LM produced by COX enzymes with potent anti-inflammatory and vasodilative effects [80]. Reduced levels of this beneficial LM could contribute to increased adipose inflammation and impaired adipose tissue blood flow (ATBF) in obesity. Dietary feeding of high-fat diet (HFD) increased tissue levels of LOX-derived leukotriene B_4 (LTB_4); and inhibition of LTB_4 receptor protected mice from HFD-induced insulin resistance and hepatic steatosis [81], suggesting that LOX pathway contributes to increased risks of obesity-associated diseases. Dietary feeding of HFD also reduced levels of CYP-derived epoxyeicosatrienoic acids (EETs), which have potent anti-inflammatory, vasodilative, and cardio-protective effects [16,82,83]. Pharmacological inhibition or transgenic deletion of soluble epoxide hydrolase (sEH, the dominant enzyme in degrading EETs) have been shown to protect mice from various adverse effects induced by obesity [38,39,70,72-76]. Together, these results support that LMs play critical roles in regulating the pathology of obesity.

Most previous studies of LMs in obesity have only studied single or limited number of LMs [38,39,70,72-76,80,81]. However, the enzymatic metabolism of PUFAs produces hundreds of LMs, which could have different or even opposite biological activities, it would be

difficult to understand the biological processes by only studying single or limited number of LMs [84]. Therefore, it is important to conduct comprehensive profiling of a variety of LMs in tissues, which could help us to better understand their roles in pathology of obesity, in order to develop novel biomarkers or therapeutic targets for obesity and obesity-associated diseases. To this end, here we used a LC-MS/MS-based lipidomics approach, which can simultaneously measure the concentrations of > 100 LMs produced by COX (COX-1, COX-2), LOX (5-LOX, 12/15-LOX) and CYP enzymes from ARA, LA, α-LA , DGLA, EPA, and DHA [85,86] (see Table 3.1), to systematically analyze how lipid signaling is deregulated in obesity.

3.3 Materials and Method

3.3.1 Obesity experiment

The animal experiment was conducted in accordance with the protocols approved by the Institutional Animal Care and Use Committee of UMass-Amherst. C57BL/6 male mice (6-week age) were maintained on a high-fat diet (60% kcal% fat, purchased from Research Diet Inc., catalog number D12492) and a control diet (10 kcal% fat, D12450J from Research Diet Inc.) for 8 weeks.

3.3.2 LC-MS/MS-based lipidomics analysis

To extract lipid metabolites from adipose tissues, ~100 mg tissues were mixed with an antioxidant solution (0.2 mg/mL butylated hydroxytoluene and 0.2 mg/mL triphenylphosphine in methanol), deuterated internal standards, and 400 μL extract solution (0.1% acetic acid with 0.2 mg/mL butylated hydroxytoluene in a methanol

solution), and then homogenized; the resulting homogenates were kept in −80 °C overnight. After centrifugation of the homogenates, the pellets were washed with methanol (containing 0.1% butylated hydroxytoluene and 0.1% acetic acid) and then combined with the supernatant. The lipid metabolites in the combined solutions were loaded onto pre-washed Waters® Oasis solid phase extraction (SPE) cartridges, washed with 95:5 water/methanol with 0.1% acetic acid, the analytes were eluted with methanol and ethyl acetate, dried using a centrifugal vacuum evaporator, then reconstituted in methanol for LC-MS/MS analysis. The LC-MS/MS analysis was carried out on an Agilent 1200SL HPLC system (Agilent, Santa Clara, CA) coupled to a 4000 QTRAP MS/MS (AB Sciex, Foster City, CA) as described in our previous report (24).

3.3.3 Real-time PCR (RT-PCR) analysis

Total RNA was isolated from gonadal adipose tissues using TRIzol Reagent (Life technologies, Carlsbad, CA) according to manufacturer's instruction. Conversion of up to 2 μg of total RNA to single stranded cDNA was preformed using High-Capacity cDNA Reverse Transcription Kit (Life technologies, Carlsbad, CA) according to manufacturer's instruction. Quantitative RT-PCR was conducted using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Agawam, MA) on a DNA Engine Opticon® 2 System (Bio-Rad Laboratories, Hercules, CA) with specific mouse primers. The primers used in this research were: Cyp2j5 (sense) 5'-TCTGGGAAGCACTCCATCTCA-3' and (antisense) 5'-CCCTGGTGGGTAGTTTTTGG-3',

Cyp2j6 (sense) 5’-TTAGCCACGATCTGGGCAG-3’ and (antisense) 5’-CTGGGGGATAGTTCTTGGGG-3’, Cyp2c44 (sense) 5’-GCTGCCCTATACAGATGCCG-3’ and (antisense) 5’-GTGACGCTAAGAGTTGCCCA-3’, Ephx2 (sense) 5’-GCGTTCGACCTTGACGGAG-3’ and (antisense) 5’-TGTAGCTTTCATCCATGAGTGGT-3’ ,
Alox15 (sense) 5’-GGCTCCAACAACGAGGTCTAC-3’ and (antisense) 5’-AGGTATTCTGACACATCCACCTT-3’, Alox5 (sense) 5’-ACTACATCTACCTCAGCCTCATT-3’ and (antisense) 5’-GGTGACATCGTAGGAGTCCAC-3’, Cox2 (sense) 5’-TTCAACACACTCTATCACTGGC-3’ and (antisense) 5’-AGAAGCGTTTGCGGTACTCAT-3’,
Pla2 (sense) 5’-TGCCTTTCCTGTAGGCTGTTC-3’ and (antisense) 5’-CGCAGGTCTCGTAGCATCTG-3’, Ptges (sense) 5’-GGATGCGCTGAAACGTGGA-3’ and (antisense) 5’-CAGGAATGAGTACACGAAGCC-3’, Ptgis (sense) 5’-ACAGCATCAAACAATTTGTCGTC-3’ and (antisense) 5’-GCATCAGACCGAAGCCATATCT-3’.

The results of target genes were normalized to β-actin gene and expressed to the control group mice using the 2-ΔΔCt method. The primer to analyze β-actin is (sense) 5’-GGCTGTATTCCCCTCCATCG-3’ and (antisense) 5’-CCAGTTGGTAACAATGCCATGT-3’.

3.3.4 Fatty acid composition analysis

Total lipids from gonadal adipose tissue were extracted as previously described (25), then treated with 3 N methanolic HCl at 55°C for 40 minutes to prepare the fatty acid methyl esters (FAMEs) (26). The resulted FAMEs dissolved in hexane were used for GC-MS analysis, using Shimadzu GC-MS-QP2010 SE (Tokyo, Japan). Oven conditions: initial temperature 50°C; temperature increase: 20°C/min to 200 °C, then increase 2°C/min to 220°C and held for 142.5 minutes. Other conditions: injector temperature 250 oC; detector temperature 250 oC; carrier gas helium, split ratio: 10:1. Column: Supelcowax 10 (fused silica), 100 m x 0.25 mm x 0.25 μm. The FAMEs were identified by comparing with the standards (Sigma-Aldrich, St. Louis, MO, or Nu-Chek Prep, Elysian, MN) or by their mass spectra, which were further compared to the NIST Mass Spectral library.

3.3.5 Data Analysis

All data are expressed as the mean ± standard error of the mean (SEM). For the comparison between the control group and HFD group, Shapiro-Wilk test was used to verify the normality of data. When data were normally distributed, statistical significance was determined using two-side t-test; otherwise, significance was determined by Mann-Whitney U test. All of these data analysis was performed by using SigmaPlot software (San Jose, CA). P values less than 0.05 are reported as statistically significant.

Table 3.1 List of eicosanoid metabolites in LC-MS/MS method.

Abbreviation	Full Name	Systematic Name
6-keto-PGF_{1a}	6-keto-Prostaglandin F1α	6-oxo-9S,11R,15S-trihydroxy-13E-prostenoic acid
20-COOH-LTB_4	12-oxo-10	(5R,6Z,8E,14Z)-5-hydroxy-12-oxoicosa-6,8,14-trienedioic acid
Resolvin E_1	Resolvin E1	5S,12R,18R-trihydroxy-6Z,8E,10E,14Z,16E-eicosapentaenoic acid
20-OH-LTB_4	20-hydroxy LTB4	(5S,6Z,8E,10E,12R,14Z)-5,12,20-trihydroxyicosa-6,8,10,14-tetraenoic acid
TXB_2	Thromboxane B2	9S,11,15S-trihydroxy-thromboxa-5Z,13E-dien-1-oic acid
PGE_3	Prostaglandin E3	9-oxo-11R,15S-dihydroxy-5Z,13E,17Z-prostatrienoic acid
PGD_3	Prostaglandin D3	9S,15S-dihydroxy-11-oxo-5Z,13E,17Z-prostatrienoic acid
9,12,13-TriHOME	Pinellic acid	9S,12S,13S-trihydroxy-10E-octadecenoic acid
9,10,13-TriHOME		9,10,13-trihydroxy-11-octadecenoic acid
PGF_{2a}	Prostaglandin F2α	9S,11R,15S-trihydroxy-5Z,13E-prostadienoic acid
PGE_2	Prostaglandin E2	9-oxo-11R,15S-dihydroxy-5Z,13E-prostadienoic acid
PGE_1	Prostaglandin E1	9-oxo-11R,15S-dihydroxy-13E-prostaenoic acid
PGD_1	Prostaglandin D1	9S,15S-dihydroxy-11-oxo-13E-prostaenoic acid
PGD_2	Prostaglandin D2	9S,15S-dihydroxy-11-oxo-5Z,13E-prostadienoic acid
LXA_4	Lipoxin A4	5S,6R,15S-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid
11,12-,15-TriHETrE	11,12,15-TriHETRE	(8E,11Z,13E)-11,12,15-trihydroxyicosa-8,11,13-trienoic acid
LTB_5	Leukotriene B5	5S,12S-dihydroxy-6Z,8E,14Z,17Z-eicosapentanenoic acid
PGJ_2	Prostaglandin J2	11-oxo-15S-hydroxy-5Z,9,13E-prostatrienoic acid
PGB_2	Prostaglandin B2	15S-hydroxy-9-oxo-5Z,8(12),13E-prostatrienoic acid
15,16-DiHODE	α-15,16-DiHODE	(+/-)-15,16-dihydroxy-9Z,12Z-octadecadienoic acid
8,15-DiHETE	8,15-DiHETE	(5Z,9E,11Z,13E)-8,15-dihydroxyicosa-5,9,11,13-tetraenoic acid
9,10-DiHODE	α-9,10-DiHODE	(+/-)-9,10-dihydroxy-12Z,15Z-octadecadienoic acid
12,13-DiHODE	α-12,13-DiHODE	(+/-)-12,13-dihydroxy-9Z,15Z-octadecadienoic acid

6-trans-LTB$_4$	6-trans-Leukotriene B4	5S,12R-dihydroxy-6E,8E,10E,14Z-eicosatetraenoic acid
5,15-DiHETE	5,15-DiHETE	(6E,8Z,11Z,13E)-5,15-dihydroxyicosa-6,8,11,13-tetraenoic acid
17,18-DiHETE	17,18-DiHETE	(+/-)-17,18-dihydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid
LTB$_4$	Leukotriene B4	5S,12R-dihydroxy-6Z,8E,10E,14Z-eicosatetraenoic acid
14,15-DiHETE	14,15-DiHETE	(+/-)-14,15-dihydroxy-5Z,8Z,11Z,17Z-eicosatetraenoic acid
11,12-DiHETE	11,12-DiHETE	(+/-)-11,12-dihydroxy-5Z,8Z,14Z,17Z-eicosatetraenoic acid
12,13-DiHOME	(9Z)-12,13-Dihydroxyoctadec-9-enoic acid	12,13-dihydroxy-9Z-octadecenoic acid
8,9-DiHETE	8,9-DiHETE	(+/-)-8,9-dihydroxy-5Z,11Z,14Z,17Z-eicosatetraenoic acid
9,10-DiHOME	Leukotoxin diol	9,10-dihydroxy-12Z-octadecenoic acid
19,20-DiHDPE	19,20-DiHDPA	(+/-)-19,20-dihydroxy-4Z,7Z,10Z,13Z,16Z-docosapentaenoic acid
14,15-DHET	(+/-)14,15-DHET	14,15-dihydroxy-5Z,8Z,11Z-eicosatrienoic acid
16,17-DiHDPE	16,17-DiHDPE	(+/-)-16,17-dihydroxy-4Z,7Z,10Z,13Z,19Z-docosapentaenoic acid
11,12-DHET	(+/-)11,12-DHET	11,12-dihydroxy-5Z,8Z,14Z-eicosatrienoic acid
13,14-DiHDPE	13,14-DiHDPE	(+/-)-13,14-dihydroxy-4Z,7Z,10Z,16Z,19Z-docosapentaenoic acid
9-HOTrE	9-HOTrE	9S-hydroxy-10E,12Z,15Z-octadecatrienoic acid
10,11-DiHDPE	10,11-DiHDPE	(+/-)-10,11-dihydroxy-4Z,7Z,13Z,16Z,19Z-docosapentaenoic acid
8,9-DHET	(+/-)8,9-DHET	8,9-dihydroxy-5Z,11Z,14Z-eicosatrienoic acid
EKODE	trans-EKODE-(E)-Ib	9-oxo-11-(3-pentyloxiran-2-yl)undec-10-enoic acid
13-HOTrE	13-HoTrE	13S-hydroxy-9Z,11E,15Z-octadecatrienoic acid
5,6-DiHETE	5S,6R-DiHETE	5S,6R-dihydroxy-7E,9E,11Z,14Z-eicosatetraenoic acid
15-deoxy-PGJ$_2$	15-deoxy-δ-12,14-Prostaglandin J$_2$	11-oxo-5Z,9,12E,14E-prostatetraenoic acid
7,8-DiHDPE	7,8-DiHDPE	(+/-)-7,8-dihydroxy-4Z,10Z,13Z,16Z,19Z-docosapentaenoic acid
20-HETE	20-hydroxy arachidonic acid	20-hydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid
15-HEPE	15-HEPE	(5Z,8Z,11Z,13E,17Z)-16-hydroxyicosa-5,8,11,13,17-pentaenoic acid
5,6-DHET	(+/-)5,6-DHET	5,6-dihydroxy-8Z,11Z,14Z-eicosatrienoic acid

8-HEPE	(+/-)-8-HEPE	(+/-)-8-hydroxy-5Z,9E,11Z,14Z,17Z-eicosapentaenoic acid
12-HEPE	(+/-)-12-HEPE	(+/-)-12-hydroxy-5Z,8Z,10E,14Z,17Z-eicosapentaenoic acid
5-HEPE	(+/-)-5-HEPE	(+/-)-5-hydroxy-6E,8Z,11Z,14Z,17Z-eicosapentaenoic acid
4,5-DiHDPE	4,5-DiHDPE	(+/-)-4,5-dihydroxy-4Z,7Z,13Z,16Z,19Z-docosapentaenoic acid
13-HODE	13S-HODE	13S-hydroxy-9Z,11E-octadecadienoic acid
9-HODE	9-HODE	9-hydroxy-10E,12Z-octadecadienoic acid
15,16-EpODE	15,16-EpODE	15,16-epoxy-13-OH-9Z,11E-octadecadienoic acid
15-HETE	15-HETE	5-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid
9,10-EpODE	9,10-EpODE	9S,10-epoxy-10,12Z-octadecadienoic acid
17,18-EEQ	17,18-EpETE	(+/-)-17(18)-epoxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid
17-HDoHE	(+/-)-17-HDoHE	(+/-)-17-hydroxy-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoic acid
11-HETE	11-HETE	11-hydroxy-5Z,8Z,11E,14Z-eicosatetraenoic acid
12,13-EpODE	α-12(13)-EpODE	12(13)-epoxy-9Z,15Z-octadecadienoic acid
13-oxo-ODE	13-KODE	13-keto-9Z,11E-octadecadienoic acid
15-oxo-ETE	15-Oxo-ETE	15-oxo-5Z,8Z,11Z,13E-eicosatetraenoic acid
9-oxo-ODE	9-OxoODE	9-oxo-10,12-octadecadienoic acid
14,15-EEQ	14(15)-EpETE	(+/-)-14(15)-epoxy-5Z,8Z,11Z,17Z-eicosatetraenoic acid
8-HETE	8-HETE	8-hydroxy-5Z,9E,11Z,14Z-eicosatetraenoic acid
12-HETE	12-HETE	12-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid
11,12-EEQ	11(12)-EpETE	(+/-)-11(12)-epoxy-5Z,8Z,14Z,17Z-eicosatetraenoic acid
8,9-EEQ	8(9)-EpETE	(+/-)-8(9)-epoxy-5Z,11Z,14Z,17Z-eicosatetraenoic acid
9-HETE	9-HETE	9-hydroxy-5Z,7E,11Z,14Z-eicosatetraenoic acid
15(S)-HETrE	15S-HETrE	15S-hydroxy-8Z,11Z,13E-eicosatrienoic acid
12-oxo-ETE	12-oxo-ETE	12-oxo-5Z,8Z,10E,14Z-eicosatetraenoic acid
5-HETE	5-HETE	5-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid
19,20-EDP	19,20-EpDPE	(+/-)-19(20)-epoxy-4Z,7Z,10Z,13Z,16Z-docosapentaenoic acid
12,13-EpOME	Vernolic acid	(+/-)-12(13)-epoxy-9Z-octadecenoic acid
14,15-EET	14,15-EpETrE	14,15-epoxy-5Z,8Z,11Z-eicosatrienoic acid
9,10-EpOME	Coronaric acid	9,10-epoxy-12Z-octadecenoic acid

16,17-EDP	16(17)-EpDPE	(+/-)-16(17)-epoxy-4Z,7Z,10Z,13Z,19Z-docosapentaenoic acid
13,14-EDP	13(14)-EpDPE	(+/-)-13(14)-epoxy-4Z,7Z,10Z,16Z,19Z-docosapentaenoic acid
5-oxo-ETE	5-Oxo-ETE	5-oxo-6E,8Z,11Z,14Z-eicosatetraenoic acid
10,11-EDP	10(11)-EpDPE	(+/-)-10(11)-epoxy-4Z,7Z,13Z,16Z,19Z-docosapentaenoic acid
11,12-EET	11,12-EpETrE	11,12-epoxy-5Z,8Z,14Z-eicosatrienoic acid
7,8-EDP	7(8)-EpDPE	(+/-)-7(8)-epoxy-4Z,10Z,13Z,16Z,19Z-docosapentaenoic acid
8,9-EET	8,9-EpETrE	8,9-epoxy-5Z,11Z,14Z-eicosatrienoic acid
5,6-EET	5,6-EpETrE	5,6-epoxy-8Z,11Z,14Z-eicosatrienoic acid

3.4 Results

3.4.1 CYP-derived LMs in adipose tissues

After 8 weeks of dietary feeding, HFD significantly increased body weight and adipose tissue weight in C57BL/6 mice (Figure 3.1). These results are consistent with previous studies of HFD on obesity [87].

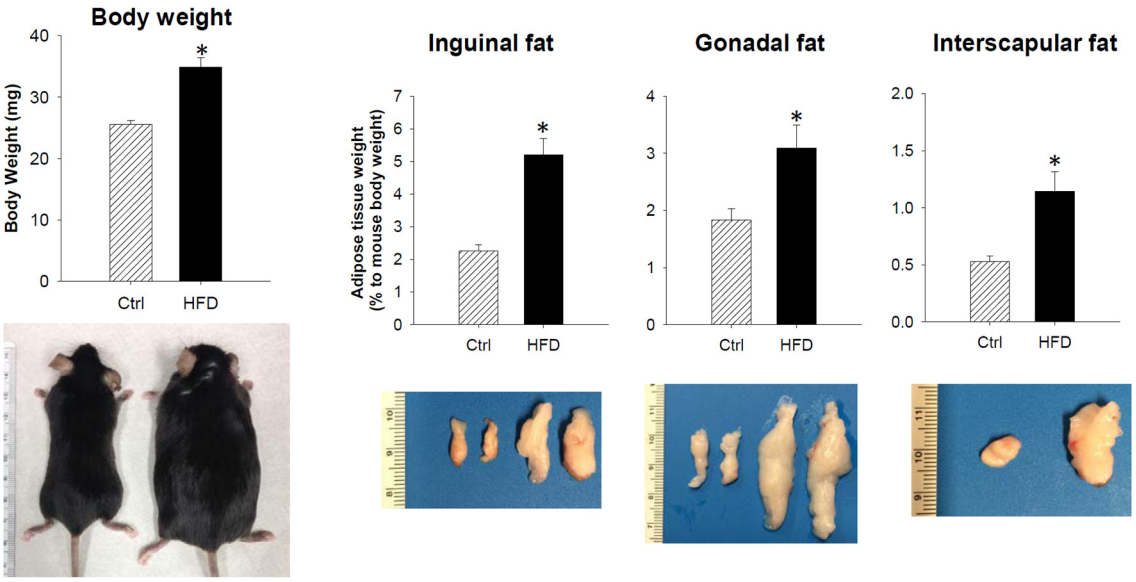

Figure 3.1 Dietary feeding of HFD for 8 weeks increased body weight and adipose tissue weight in C57BL/6 mice.

We used LC-MS/MS-based lipidomics to compare the profiles of LMs in inguinal, gonadal, and interscapular adipose tissues of mice fed on HFD or control diet. Among the three major PUFA metabolizing pathways (COX, LOX, and CYP), CYP-derived fatty acid epoxides are the most dramatically altered LMs in adipose tissues. The levels of fatty acid epoxides derived from several PUFAs, including epoxyoctadecenoic acids (EpOMEs) derived from LA, EETs from ARA, epoxydocosapentaenoic acids (EpDPEs) from DHA, and epoxyoctadecadienoic acids (EpODEs) from α-LA, were significantly reduced in different adipose tissues (inguinal, gonadal, and interscapular fats) of HFD-fed mice (Figure 3.2A-C). For example, the concentrations of 11,12-EET and 14,15-EET were reduced by respective 88±20% and 89±18% in inguinal adipose tissues ($P \leq 0.001$), and were reduced by respective 88±13% and 89±11% in gonadal fat tissues of HFD-fed mice ($P \leq 0.001$).

The fatty acid epoxides are further metabolized by soluble epoxide hydrolase (sEH) to generate the corresponding fatty acid diols [81]. Consistent with reduced adipose levels of fatty acid epoxides, the levels of fatty acid diols were also significantly reduced (Figure 3.2A-C). For example, the levels of 11,12-dihydroxyeicosatrienoic acid (11,12-DHET), which is a sEH metabolite of 11,12-EET, were reduced by 54.5±10.7% (mean ± SEM) in gonadal adipose tissues of HFD-fed mice ($P \leq 0.001$). We need to point out in the adipose tissues, the concentrations of fatty acid epoxides were much higher than those of the corresponding fatty acid diols. For example, the concentration of 11,12-EET was ~73-fold higher than its sEH metabolite 11,12-DHET in gonadal adipose tissues of control mice.

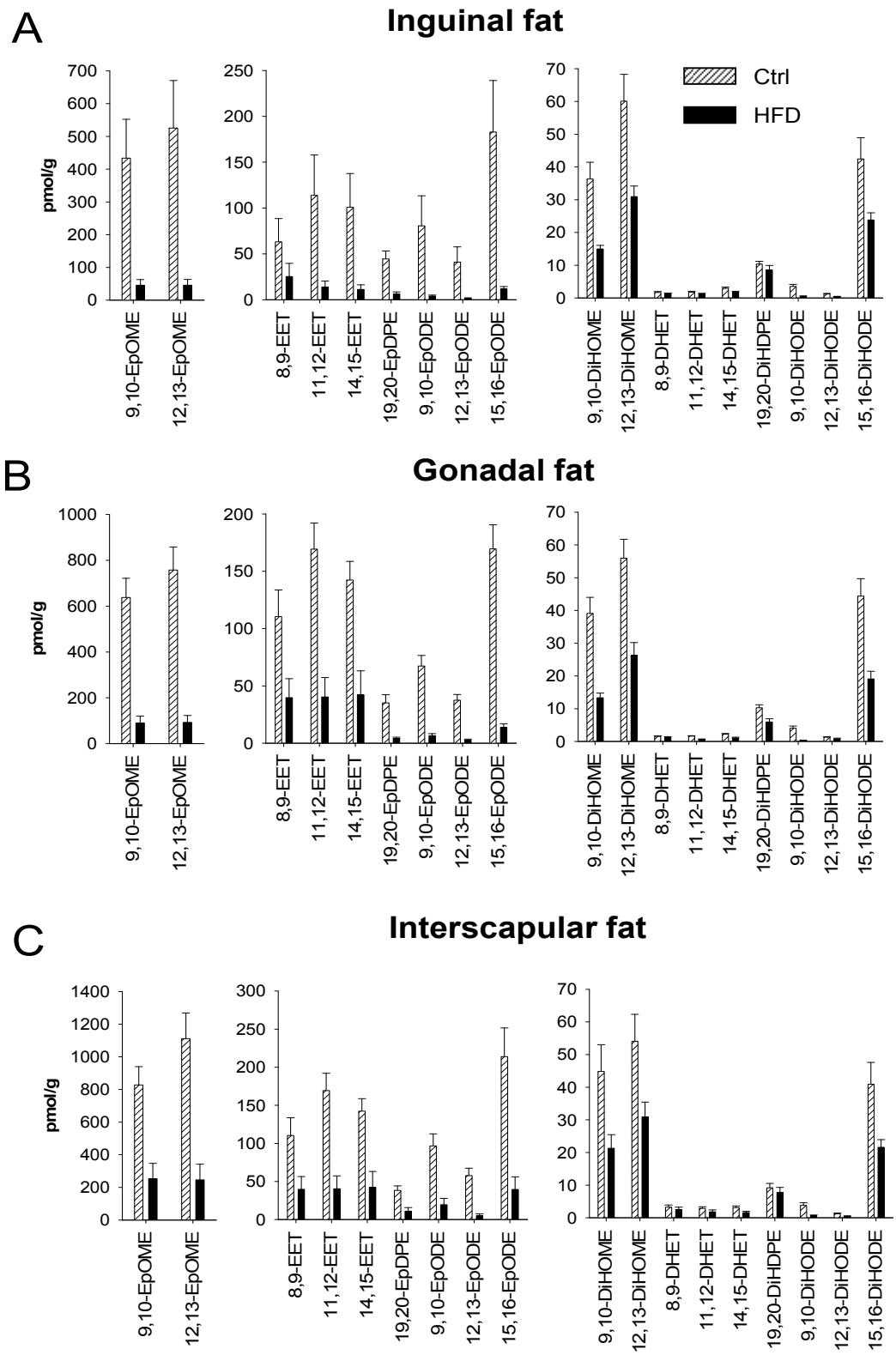

Figure 3.2 Dietary feeding of HFD reduces levels of CYP-derived fatty acid epoxides and fatty acid diols in adipose tissues.

(A) profiles of CYP-derived LMs in inguinal adipose tissues. (B) profiles of CYP-derived LMs in gonadal adipose tissues. (C) profiles of CYP-derived LMs in interscapular adipose tissues.

3.4.2 COX-derived LMs in adipose tissues

The metabolism of PUFAs by COX enzymes lead to formation of prostaglandin H_2 (PGH_2), which was further enzymatically metabolized to generate various prostaglandins (Figure 3.3A). Compared with CYP-derived LMs, the profiles of COX-derived LMs showed more complicated patterns. In inguinal adipose tissues, the levels of COX-derived LMs were not changed (Figure 3.3B), suggesting that COX pathway is not likely to be involved in the biology of inguinal adipose tissues. In gonadal adipose tissues, the concentrations of PGI_2 (as measured by its stable metabolite 6-keto-PGF_{1a}) and PGE_2 were significantly reduced in HFD-fed mice (Figure 3.3C). In interscapular adipose tissues, the concentrations of $PGF_{2\alpha}$ and PGE_2 were significantly reduced in HFD-fed mice ($P < 0.05$, Figure 3.3D).

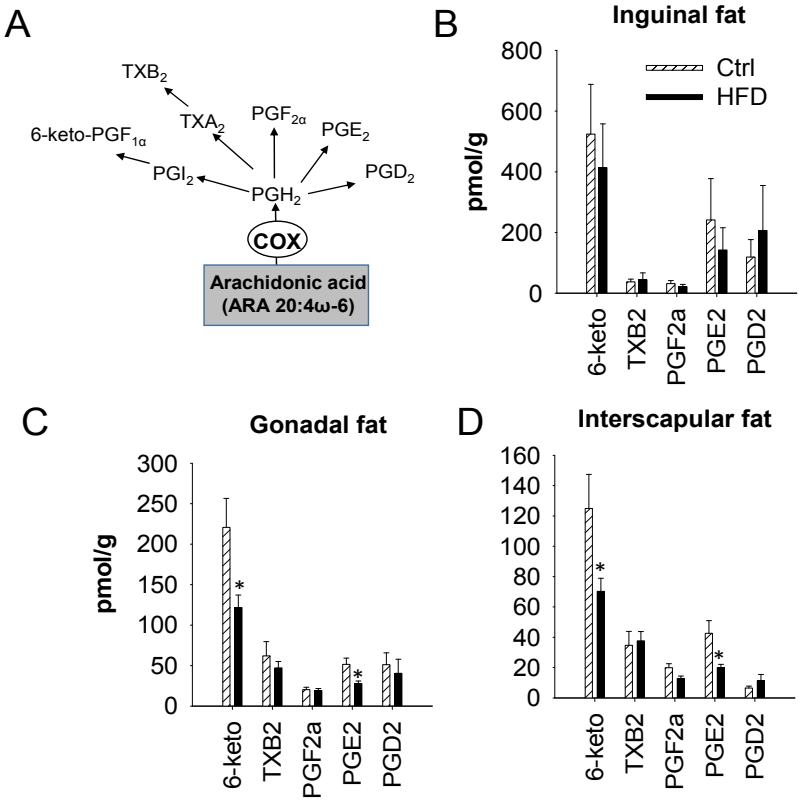

Figure 3.3 Dietary feeding of HFD modulates COX-derived LMs in adipose tissues. (A) a simplified sheme of COX-2 pathway. (B) profiles of COX-derived LMs in inguinal adipose tissues. (C) profiles of COX-derived LMs in gonadal adipose tissues. (D) profiles of COX-derived LMs in interscapular adipose tissues.

3.4.3 5-LOX-derived LMs in adipose tissues

The metabolism of ARA by 5-LOX leads to formation of 5-hydroperoxyeicosatetraenoic acid (5-HpETE), which is then converted to at least two classes of LMs: (1) 5-hydroxyeicosatetraenoic acid (5-HETE), or similar LMs such as α-LA-derived 9-HOTrE and EPA-derived 5-HEPE, and (2) LTB_4, or similar LMs such as EPA-derived leukotriene B_5 (LTB_5) (Figure 3.4A). Dietary feeding of HFD reduced levels of 5-HETE-series LMs (such as 5-HETE, 9-HOTrE, and 5-HEPE), while increased levels of LTB_4 in adipose tissues (Figure 3.4B-D). The tissue levels of LTB_4 were significantly increased in

inguinal, gonadal, interscapular adipose tissues of HFD-fed mice (Figure 3.4B-D), which is in agreement with studies which showed that HFD increased tissue levels of LTB_4 [81].

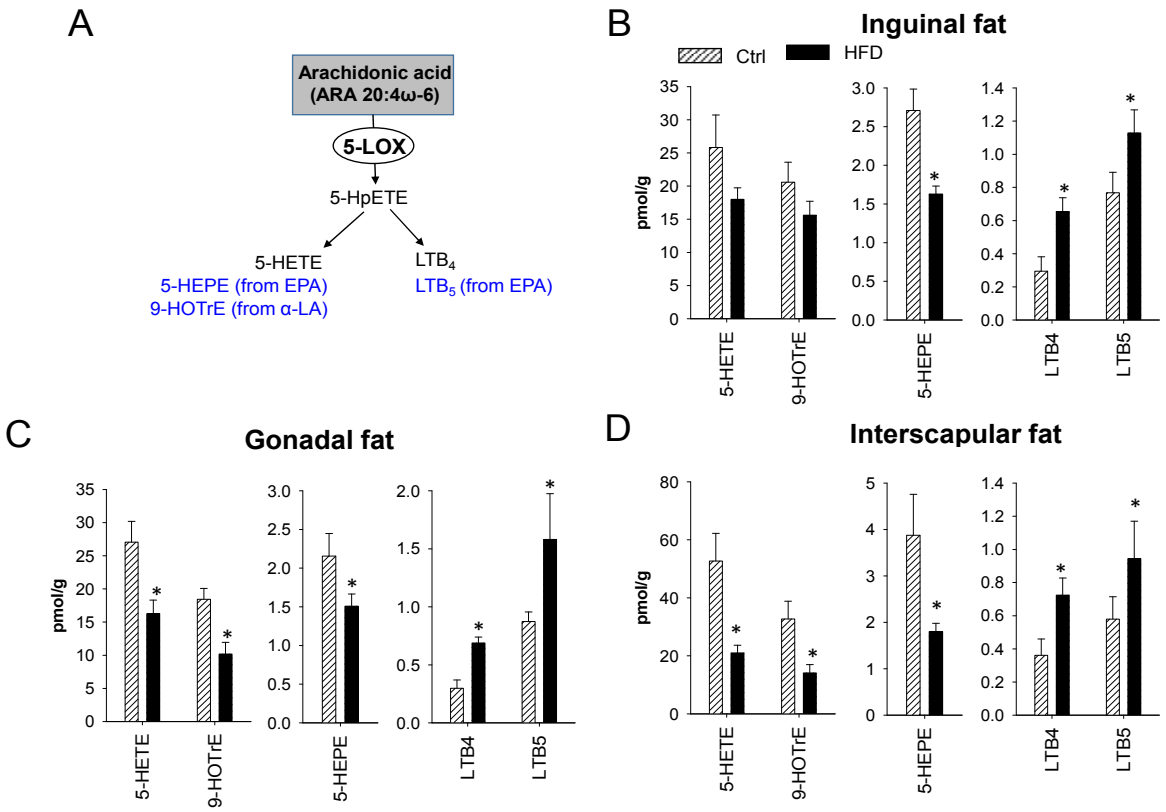

Figure 3.4 Dietary feeding of HFD modulates 5-LOX-derived LMs in adipose tissues.

(A) a simplified sheme of 5-LOX pathway. (B) profiles of 5-LOX-derived LMs in inguinal adipose tissues. (C) profiles of 5-LOX-derived LMs in gonadal adipose tissues. (D) profiles of 5-LOX-derived LMs in interscapular adipose tissues.

3.4.4 12/15-LOX-derived LMs in adipose tissues

The metabolism of PUFAs by 12/15-LOX leads to formation of a series of LMs (Figure 3.5A). LC-MS/MS showed that many of these LMs were reduced in adipose tissues of HFD-fed mice (Figure 3.5B-D). For example, the tissue levels of 15-HETE were significantly reduced in inguinal, gonadal, and interscapular adipose tissues of HFD-fed mice (Figure 3.5B-D). 12-HETE, which is among the most abundant LOX-derived LMs

in adipose tissues, was reduced by 71.7±6.78% (mean ± SEM) in interscapular adipose tissue of HFD-fed mice (Figure 3.5D).

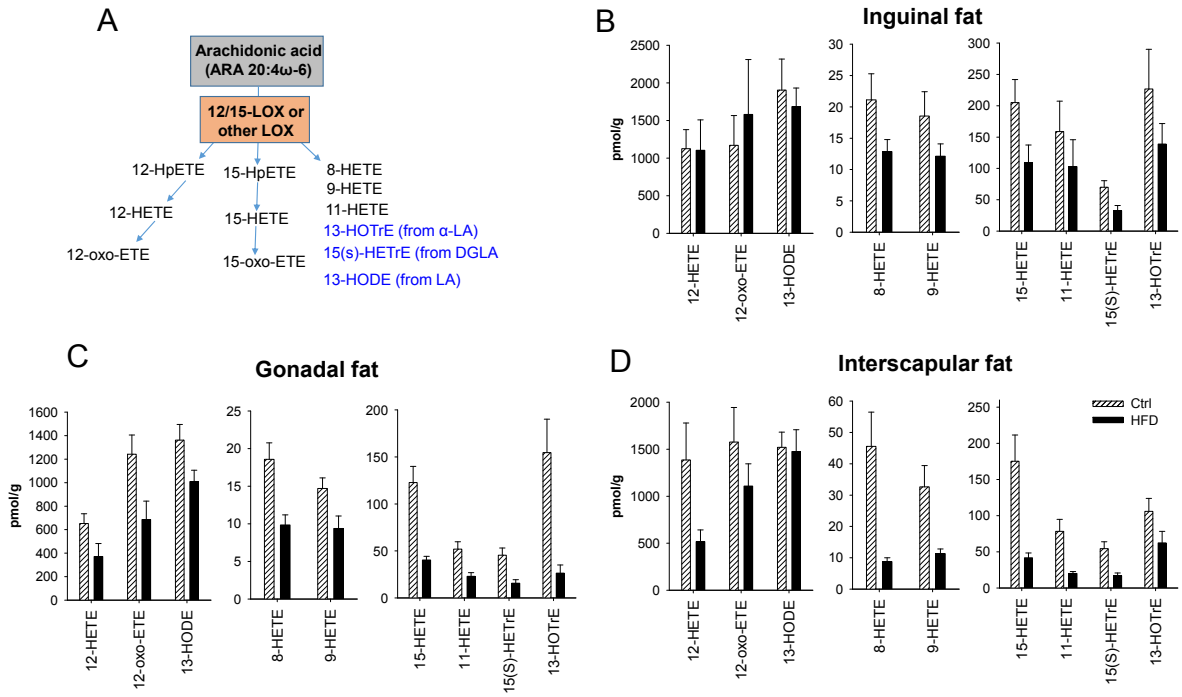

Figure 3.5 Dietary feeding of HFD modulates 12/15-LOX-derived LMs in adipose tissues.

(A) a simplified sheme of 12/15-LOX pathway. (B) profiles of 12/15-LOX-derived LMs in inguinal adipose tissues. (C) profiles of 12/15-LOX-derived LMs in gonadal adipose tissues. (D) profiles of 12/15-LOX-derived LMs in interscapular adipose tissues.

3.4.5 Fatty acid composition and expression of COX, LOX and CYP in adipose tissues

The tissue profiles of LMs are in part mediated by fatty acid composition and expression of PUFA metabolizing enzymes in the tissues [3]. To understand the mechanisms by which HFD modulated LMs in adipose tissues, we analyzed fatty acid composition and gene expression of COX, LOX and CYP in adipose tissues. We focused on gonadal adipose tissues, which are the largest adipose tissues, since we have observed significant changes of most LMs in gonadal fat (Figure 3.1-3.5).

For fatty acid composition, as expected, triglycerides were major lipids in the gonadal adipose tissue, with minimum amount of phospholipids (determined by TLC method based on Ref [88], data not shown), which is consistent to previous studies [89]. Therefore, we analyzed fatty acid composition from the total lipid of adipose tissues. GC-MS analysis showed that dietary feeding of HFD did not change the tissue levels of ARA and α-LA, and slightly increased tissue levels of LA (22.50±0.18% in HFD group *vs.* 17.78±0.44% in control group, $P < 0.001$). These results support that dietary feeding of HFD did not reduce levels of PUFAs in adipose tissues, suggesting that the reduced levels of many LMs in adipose tissues were not due to lack of PUFA substrates.

For gene expressions of PUFA metabolizing enzymes, RT-PCR showed that the expressions of several CYP monooxygenases, such as *Cyp2j5*, *Cyp2j6*, and *Cyp2c44*, were significantly reduced in the gonadal adipose tissues of HFD-fed mice (Figure 3.6A), which is well consistent with the reduced levels of CYP-derived fatty acid epoxides in adipose tissues (see Figure 3.6B). The expression of *Ephx2* (encoding sEH) was not changed (Figure 3.6A). For COX pathway, the gene expression of *Cox2* was not changed, while the expressions of *Ptges* (encoding microsomal prostaglandin E synthase) and *Ptgis* (encoding PGI_2 synthase) were significantly reduced in HFD-fed mice (Figure 3.6B). This is consistent with the LC-MS/MS analysis which showed that only PGE_2 and PGI_2, but not other COX-derived LMs, were reduced in adipose tissues of HFD-fed mice. For LOX pathways, the gene expressions of *Alox15* and *Alox5* (encoding 5-LOX) were not significantly changed (Figure 3.6C), suggesting that the effects of HFD on LOX-derived LMs may be though modulations of down-stream enzymes. Finally, we analyzed the

expression of *Pla2*, and found little change of this gene (Figure 3.6C). Together, these results support that HFD changed tissue profiles of LMs mainly through modulation of the expressions of PUFA metabolizing enzymes in adipose tissues.

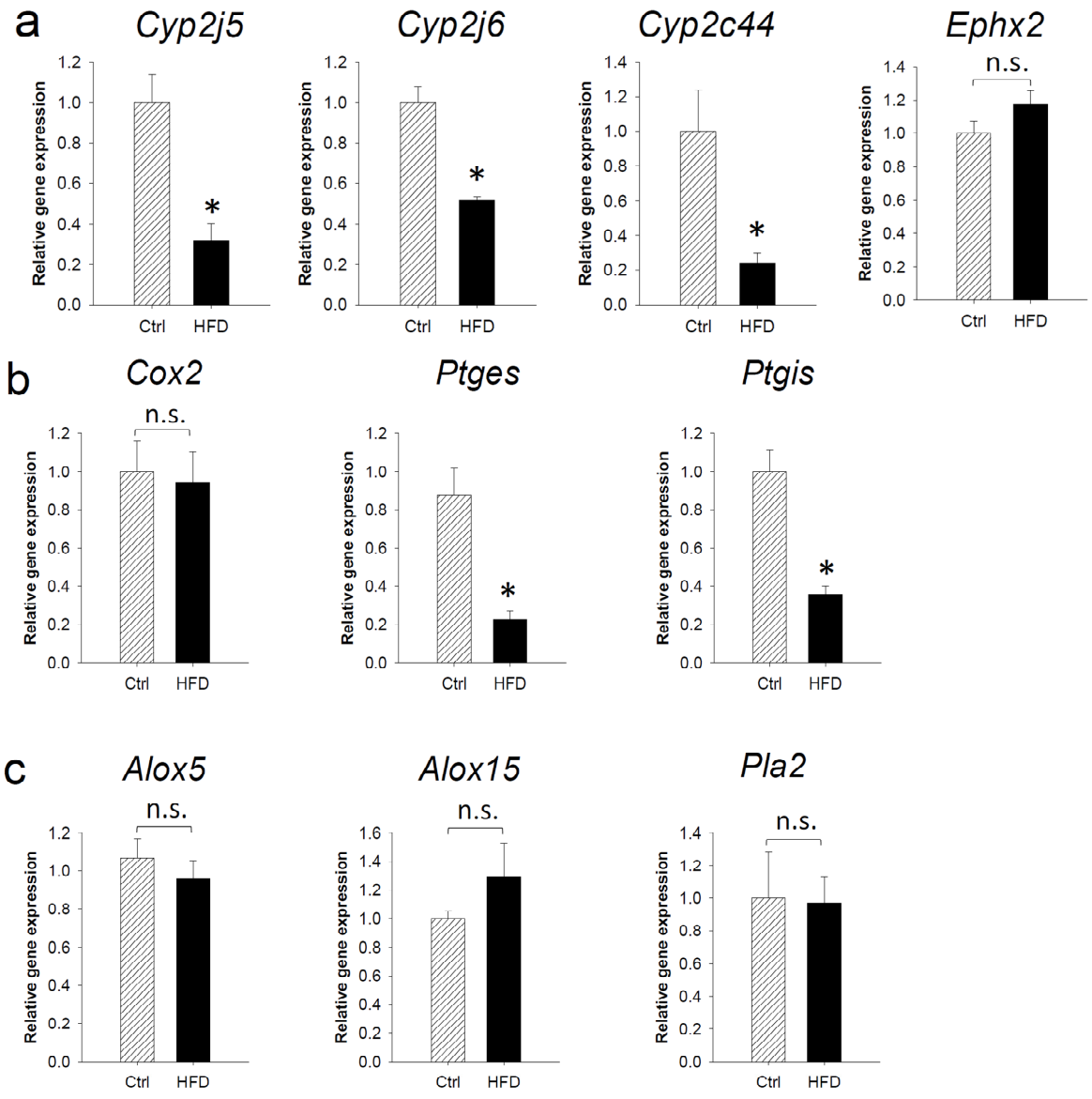

Figure 3.6 Effect of HFD on gene expresions of PUFA metabolizing enzymes in gonadal adipose tissues.

(A) gene expressions of enzymes involved in CYP pathway. (B) gene expressions of enzymes in COX pathway. (C) gene expressions of enzymes in LOX pathway, as well as phospholipase A2 (PLA2).

3.5 Discussion

In this study, we conducted a LC-MS/MS-based lipidomics analysis of HFD-induced obesity in mice. Our central finding is that HFD significantly modulated the profiles of LMs in adipose tissues of mice. Among the three major PUFA metabolizing pathways (COX, LOX and CYP), CYP-derived fatty acid epoxides are the most dramatically changed LMs in adipose tissues of HFD-induced obesity. Almost all types of fatty acid epoxides, including EpOMEs derived from LA, EETs from ARA, EpDPEs from DHA, and EpODEs from α-LA, were reduced by 70-90% in different types of adipose tissues. Based on our GC-MS analysis of fatty acid composition and RT-PCR analysis of PUFA metabolizing enzymes, these changes were most likely caused by reduced expressions of CYP monooxygenases, not because the PUFA substrates were reduced in adipose tissues of HFD-fed mice. Our results are consistent with previous studies, which showed that EETs are reduced in adipose tissues of HFD-fed mice [16]. Many of these fatty acid epoxides have beneficial effects on health. ARA-derived EETs have been shown to have potent anti-inflammatory, vasodilative, anti-hypertensive, cardio-protective, renal-protective, and analgesic actions [90]. DHA-derived EpDPEs have been shown to be the most potent fatty acid epoxides in dilation of blood vessels, with EC_{50} values of 0.5- 24 pM for dilation of porcine coronary arterioles [91]. Our own study has shown that EDPs have potent anti-angiogenic, anti-cancer and anti-metastatic effects *in vitro* and *in vivo* [51]. Therefore, reduced levels of these beneficial LMs, in particular EETs and EDPs, may contribute to the adverse effects of obesity. This is supported by recent studies, which showed that pharmacological inhibition or transgenic deletion of sEH, which is the dominant enzyme in degrading fatty acid epoxides, protected mice form various adverse

consequences of obesity, such as endoplasmic reticulum stress, metabolic syndrome, hepatic steatosis, inflammation, and endothelial dysfunction [16,38,39,70,72-76]. Together, these results strongly support that CYP-derived fatty acid epoxides play important roles in regulating pathology of obesity.

The profiles of COX- and LOX-derived LMs showed more complicated pattern in adipose tissues of HFD-induced obesity. For COX pathway, the relative balance of vasodilative PGI_2 (as measured by its stable metabolite 6-keto-PGF_{1a}) and vasoconstrictive TXA_2 (as measured by its stable metabolite TXB_2) plays critical role in regulating vascular tone and cardiovascular functions [1,92]. Our study showed that dietary feeding of HFD reduced adipose levels of vasodilative PGI_2, while had little effect on vasoconstrictive TXA_2. These results support that PGI_2 pathway, but not TXA_2 pathway, may contribute to some adverse effects of obesity. Our results are consistent with previous studies which showed that biosynthesis of PGI_2, but not TXA_2, was attenuated in obese Zucker rats [80]. We also found that the tissue levels of PGE_2 were significantly reduced in obese mice. This is consistent with previous studies of HFD on adipose tissue levels of PGE_2 [93-96]. The biological significance of PGE_2 remains to be determined. On one hand, PGE_2 is a potent vasodilator, reduced level of PGE_2 could contribute to reduced ATBF of obesity [1]; on the other hand, PGE_2 is a potent inducer of inflammation [1], reduced level of PGE_2 is not consistent with the enhanced adipose inflammation in obesity. Previous studies have shown that HFD induced a dynamic change of adipose level of PGE_2: in the early stage of HFD feeding (day 4 post HFD feeding), PGE_2 was increased in adipose tissues; while at a later stage (day 14), its concentration was reduced

in adipose tissues [94]. These results support that there may be a highly time-dependent change of tissue levels of LMs, in order to respond to varied cellular stimulations at different stages of obesity development.

For LOX pathway, only the concentration of 5-LOX-derived LTB_4 was significantly increased in adipose tissues. This is consistent with recent studies which showed that HFD increased tissue levels of LTB_4; in addition, inhibition of LTB_4 receptor protected mice from HFD-induced insulin resistance and hepatic steatosis [81], supporting a critical role of LTB_4 in pathology of obesity. For 12/15-LOX pathway, our results showed that the many 12/15-LOX-derived LMs (12-HETE, 15-HETE, 15(s)-HETrE, and 13-HOTrE) were reduced in adipose tissues of HFD-fed mice. Our results are consistent with previous studies which showed that HFD reduced adipose concentrations of 12-HETE and 15-HETE [96]. Some previous studies have shown that 12/15-LOX pathway is activated in obesity [97]; and these different results could be because different animal models of obesity are used. Many of the LOX-derived hydroxyl fatty acids and leukotrienes have potent effects to regulate inflammation and vascular tone [1]. It remains to determine whether reduced levels of 12/15-LOX-derived LMs contributed to adverse effects of obesity.

In conclusion, our lipidomics analysis showed that HFD significantly modulated the profiles of LMs in adipose tissues of mice. In particular, CYP-derived fatty acid epoxides are the most dramatically altered LMs in HFD-induced obesity, suggesting that these novel LMs could play critical roles in pathology of obesity. This lipidomics study lays

the foundation to further investigate the functional roles of LMs in obesity, which could facilitate the development of novel biomarkers or therapeutic targets for obesity and obesity-associated diseases.

CHAPTER 4

TARGETED METABOLOMICS IDENTIFIES CYTOCHROME P450 MONOOXYGENASE EICOSANOID PATHWAY AS NOVEL THERAPEUTIC TARGET OF COLON TUMORIGENESIS

4.1 Abstract

Colon cancer is the third most common cancer and the second leading cause of cancer-related death in the United States, emphasizing the need for discovery of novel cellular targets which are crucial in the pathogenesis of colon cancer. Using a metabolomics approach, we find that epoxygenated fatty acids (EpFAs), which are eicosanoid metabolites produced by cytochrome P450 (CYP) monooxygenases, are increased in both the plasma and colon of azoxymethane (AOM)/dextran sodium sulfate (DSS)-induced colon cancer mice. We further find that CYP monooxygenases are overexpressed in colon tumor tissues and colon cancer cells. Pharmacological inhibition or genetic ablation of CYP monooxygenases suppresses AOM/DSS-induced colon tumorigenesis *in vivo*. In addition, treatment with 12,13- epoxyoctadecenoic acid (EpOME), which is a metabolite of CYP monooxygenase produced from linoleic acid, increases cytokine production and JNK phosphorylation *in vitro*, and exacerbates AOM/DSS-induced colon tumorigenesis *in vivo*. Together, these results demonstrate that the previously unappreciated CYP monooxygenase pathway is upregulated in colon cancer, contributes to its pathogenesis, and could be therapeutically explored for preventing or treating colon cancer.

4.2 Introduction

Colon cancer is the third most common cancer and the second leading cause of cancer-related death in the United States [6]. It is important to discover novel cellular targets which are crucial in the pathogenesis of colon cancer, which could facilitate development of

mechanism-based strategies to reduce the risks of colon cancer. Eicosanoids, which are endogenous lipid signaling molecules produced from enzymatic metabolism of arachidonic acid (ARA, 20:4ω-6), play essential roles in inflammation and have recently been implicated in cancer [18,19]. There are three major pathways involved in biosynthesis of eicosanoids: cyclooxygenases (COX) producing prostaglandins and thromboxanes, lipoxygenases (LOX) producing leukotrienes and hydroxyl fatty acids, and cytochrome P450 (CYP) monooxygenases producing epoxygenated fatty acids (EpFAs) [1,3]. Besides ARA, other polyunsaturated fatty acids, such as linoleic acid (LA, 18:2ω-6), α-linolenic acid (ALA, 18:3ω-3), and docosahexaenoic acid (DHA, 22:6ω-3), are also efficient alternative substrates for these metabolizing enzymes, which convert them to the corresponding fatty acid metabolites [1,3]. As a result, the enzymatic metabolism of polyunsaturated fatty acids leads to formation of a large array of eicosanoid metabolites, many of which act as autocrine or paracrine mediators to regulate inflammation and hemostasis [1,3].

Previous research has shown that certain eicosanoid metabolites are deregulated in colon cancer and contribute to its pathogenesis [23]. Notably, the most prominent cancer-associated eicosanoids are prostaglandins, which are produced by COX-2 that is overexpressed in most human colon cancer samples [19]. Clinical and epidemiological studies support that pharmacological inhibitors of COX-2 are highly effective for preventing colon cancer [19], however, the gastrointestinal and cardiovascular toxicities induced by COX-2 inhibitors have limited their clinical applications [22,98]. Besides COX-2, the roles of other eicosanoid pathways in colon tumorigenesis are not well understood

[23]. It is important to discover novel eicosanoid signaling pathways involved in colon tumorigenesis.

Most previous studies to investigate the roles of eicosanoids in colon tumorigenesis have only studied single or limited number of eicosanoid metabolite(s), few systematic analyses have been carried out, hampering our understanding of the roles of eicosanoid signaling in colon tumorigenesis [23]. To discover novel eicosanoid metabolites and pathways involved in colon tumorigenesis, in this study we use a liquid chromatography tandem-mass spectrometry (LC-MS/MS)-based targeted metabolomics to systematically profile eicosanoids in a well-stablished azoxymethane (AOM)/dextran sulfate sodium (DSS)-induced colon cancer model in C57BL/6 mice, then use pharmacological and genetic approaches to validate the functional roles of identified metabolite in colon tumorigenesis.

4.3 Materials and Method

4.3.1 Animal experiment

The animal experiments were conducted in accordance with the protocols approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Amherst (protocol number: 2017-0019) and National Institutes of Health/National Institute of Environmental Health Sciences (protocol number: 05-18).

Animal Protocol 1: AOM/DSS-induced colon tumorigenesis in mice

C57BL/6 male mice (age = 6 weeks) were purchased from Charles River and maintained on a modified AIN-93G diet (containing 10% fat, corn oil was the only source of fat content, diet composition was described in Table 4.1) for 3 weeks, then the mice were divided into two groups: (1) the mice in the AOM/DSS group were treated with 10 mg/kg AOM (Sigma-Aldrich) via intraperitoneal injection, at week 1 post the AOM injection, they were given 2% DSS (36–50 kDa, MP Biomedicals) in drinking water for 1 week; and (2) the mice in control group were maintained on the same diet without AOM/DSS treatment. At week 9.5 post the AOM injection, the mice were sacrificed to harvest plasma and colon tissues for analysis. For analysis of colon tumor, colon tissues were cut open longitudinally, washed in PBS and inspected under a dissection microscope. The size of the tumor was determined by the following formula: tumor size = $\pi \times d^2/4$, where d is the diameter of each tumor.

Animal Protocol 2: AOM/DSS-induced colon tumorigenesis in *Cyp2c*$^{+/+}$, *Cyp2c*$^{+/-}$ and *Cyp2c*$^{-/-}$mice

Littermate wildtype *Cyp2c*$^{+/+}$ mice, heterozygous *Cyp2c*$^{+/-}$ mice and knockout *Cyp2c*$^{-/-}$ mice (background on C57BL/6, age = 6 weeks) were treated with 10 mg/kg AOM via i.p. injection. At week 1 post the AOM injection, they were given 2% DSS in drinking water for 1 week. During the whole experiment, the mice were maintained on mouse chow. At week 9.5 post the AOM injection, the mice were sacrificed to collect blood and colon tissues for analysis.

Animal Protocol 3: Effects of 12,13-epoxyoctadecenoic acid (EpOME) on AOM/DSS-induced colon tumorigenesis

C57BL/6 male mice (age = 6 weeks) were treated with 10 mg/kg AOM via i.p. injection. At week 1 post the AOM injection, they were given 2% DSS in drinking water for 1 week. At week 5 post the AOM injection, the mice were subcutaneously implanted with osmotic mini-pumps (Durect), which contained 12,13-EpOME (Cayman, dose of 12,13-EpOME = 2 mg/kg/day) or vehicle (a 1:1 vol/vol mixed solution of DMSO and PEG 400). During the whole experiment, the mice were maintained on mouse chow. At week 9 post the AOM injection, the mice were sacrificed to collect blood and colon tissues for analysis.

Animal Protocol 4: Effects of CYP inhibitors (SKF-525A and clotrimazole) on AOM/DSS-induced colon tumorigenesis in mice

C57BL/6 male mice (age = 6 weeks, maintained on mouse chow) were treated with 10 mg/kg AOM via i.p. injection. At week 1 post the AOM injection, they were given 2% DSS in drinking water for 1 week. After the AOM/DSS stimulation, the mouse diet was changed to a modified AIN-93G diet containing 200 ppm SKF-525A or clotrimazole (Alfa Aesar) dissolved in polyethylene glycol 400 (PEG 400, Millipore) as vehicle (0.1% in diet, v/w), or vehicle alone for 6 weeks. The diets were freshly prepared and changed every 2-3 days. At week 8 post the AOM injection, the mice were sacrificed to collect blood and colon tissues for analysis.

4.3.2 Statistical analysis

Data are expressed as means ± SEM. Shapiro–Wilk test was used to verify the normality of data and Levene's mean test was used to assess equal variance of data. Statistical comparison of two groups was performed using either Student's t test or Wilcoxon-Mann-Whitney test (when normality test is failed), comparison of three groups was analyzed by either parametric one-way ANOVA or nonparametric one-way ANOVA (Kruskal–Wallis test by ranks, used when normality test is failed) followed by Dunn's post hoc test. All of these data analysis was performed by using SigmaPlot software (Systat Software, Inc). P values less than 0.05 are reported as statistically significant.

Table 4.1 Composition of the modified AIN-93G diet used in the animal experiment

Ingredients	**grams/kg**
Casein	200
L-Cystine	3
Sucrose	100
Dyetrose	132
Cornstarch	397.5
Cellulose	50
Mineral mix #210025	35
Vitamin mix #310025	10
Choline Bitartrate	2.5
Corn oil	100

4.4 Results

4.4.1 CYP monooxygenase-produced eicosanoid metabolites are elevated in the plasma and colon of AOM/DSS-induced colon cancer mice

To our knowledge, a metabolomics-based approach to systematically profile eicosanoids in colon cancer has not been attempted [23]. In an effort to better understand the roles of eicosanoids in colon tumorigenesis, we used a LC-MS/MS-based targeted metabolomics to compare the profiles of eicosanoids in the plasma and colon of control healthy mice (not treated with AOM/DSS) and colon cancer mice (treated with AOM/DSS) (Figure 4.1A). In agreement with previous studies of the AOM/DSS model [99], 100% of the AOM/DSS-treated mice developed colon tumors (Figure 4.1B), with increased expressions of pro-inflammatory genes (*Il-6*, *Tnf-α*, *Mcp-1*, and *Cox-2*) in the colon tissues (Figure 4.1C).

LC-MS/MS detected 42 metabolites in the plasma and 56 metabolites in the colon. Among the detected metabolites, only CYP monooxygenase-produced EpFAs are significantly increased in both the plasma and colon tissues of the AOM/DSS-induced colon cancer mice (Figure 4.1D-G). Indeed, the concentrations of an array of EpFAs, including 9,10- and 12,13-epoxyoctadecenoic acid (EpOME) produced from LA (Figure 4.1D), 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acids (EET) from ARA (Figure 4.1E), 9,10- and 15,16-epoxyoctadecadienoic acid (EpODE) from ALA (Figure 4.1F), and 19,20-epoxydocosapentaenoic acid (EDP) from DHA (Figure 4.1G), are increased in the plasma and colon of the tumor-bearing mice.

4.4.2 CYP monooxygenases are overexpressed in the colon of AOM/DSS-induced colon cancer mice

To understand the underlying mechanisms behind elevated concentrations of EpFAs in colon cancer, we studied the expression of enzymes involved in EpFA production and removal. The biosynthesis and degradation of EpFAs involves three enzymatic steps: membrane-bound fatty acids are released by phospholipase A_2 (PLA_2) and related enzymes to generate intracellular free-form fatty acids, which are then metabolized by CYP monooxygenases (predominately CYP2C isoforms) to form EpFAs, which then undergo degradation by soluble epoxide hydrolase (sEH) [2,3]. qRT-PCR showed that the expression of *Pla2g4a* (encoding cytosolic calcium-dependent PLA_2) was unchanged, the expression of several *CYP* monooxygenases (including mouse *Cyp2c38*, *Cyp2c39*, *Cyp2c65*, and *Cyp2c70*) was increased ($P < 0.05$), and the expression of *Ephx2* (encoding sEH) was decreased ($P < 0.05$), in colon tissue of mice with colon cancer (Figure 4.1H). Together, these results support that there is enhanced biosynthesis and reduced degradation of EpFAs in colon tumors of mice, which contributes to elevated EpFAs in colon cancer.

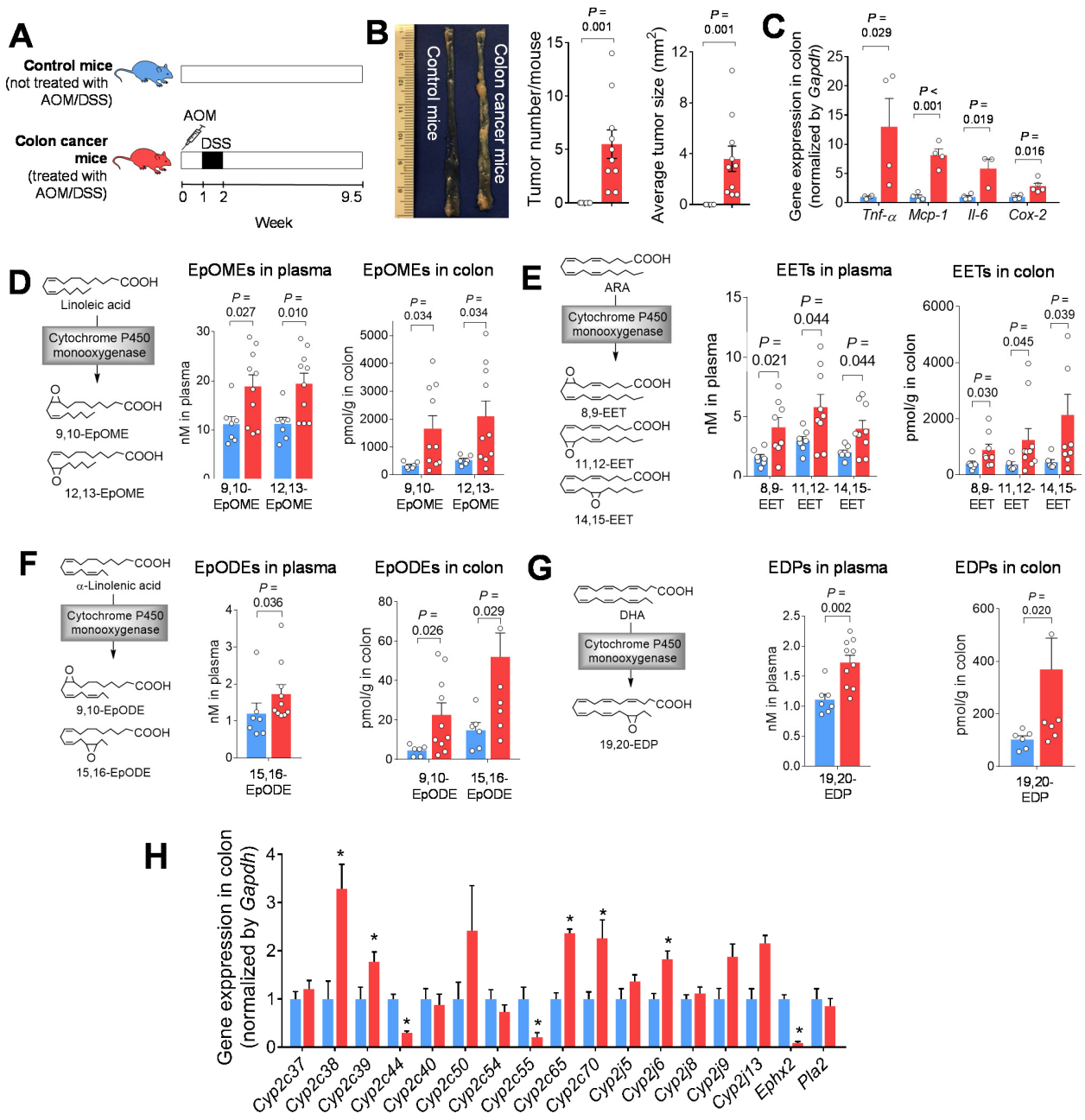

Figure 4.1 CYP monooxygenase-produced eicosanoid metabolites are increased in the plasma and colon of AOM/DSS-induced colon cancer mice.

(A) Scheme of animal experiment. (B) Quantification of colon tumor in mice. (C) Gene expression of Tnf-α, Mcp-1, Il-6 and Cox-2 in colon. (D-G) Concentrations of CYP monooxygenase-produced EpFAs in plasma and colon. (H) Expression of Pla2, Cyp monooxygenases, and Ephx2 (encoding sEH) in colon.

4.4.3 CYP monooxygenases are overexpressed in human colon cancer cells

To further validate that CYP monooxygenases are overexpressed in colon cancer, we studied their expressions in human colon cancer cells. Compared with normal human colon cells (CCD-18Co), the gene expression of *CYP* monooxygenases (human *CYP2C8*, *2C9*, *2C19*, and *2J2*) was significantly ($P < 0.05$) increased in human colon cancer cells (HCT116 and Caco-2) (Figure 4.2A). Consistent with the qRT-PCR result, immunoblotting showed that the protein expression of CYP monooxygenase (using human CYP2C9 as a marker) was increased in human colon cancer cells (Figure 4.2B). These results demonstrate that the CYP monooxygenases are overexpressed in human colon cancer cells, which is consistent with the results from animal experiments.

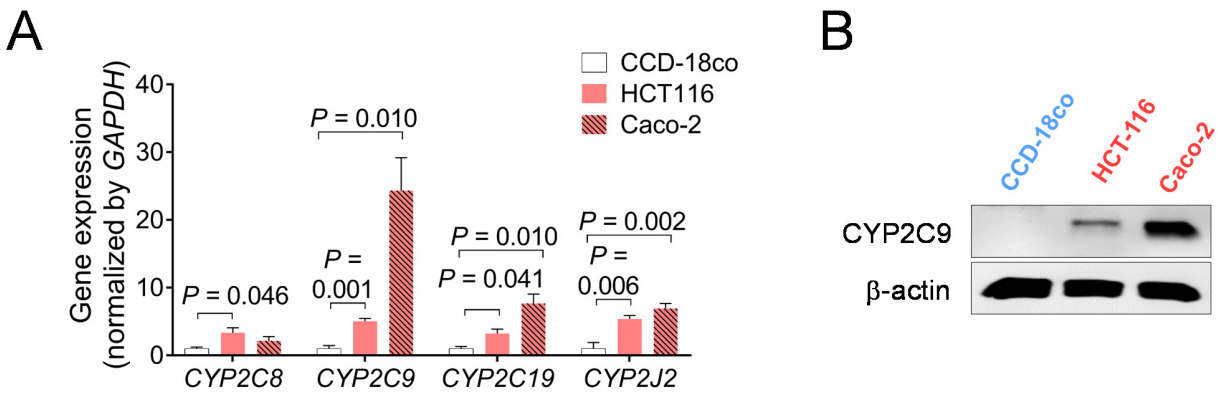

Figure 4.2 CYP monooxygenases are overexpressed in human colon cancer cells. (A) Gene expression of CYP monooxygenases (human CYP2C8, CYP2C9, CYP2C19, and CYP2J2) in normal human colon cells (CCD-18Co) and human colon cancer cells (HCT-116 and Caco-2). (B) Western blotting analysis of CYP2C9 expression in normal colon cells and colon cancer cells.

4.4.4 Genetic ablation of CYP monooxygenases suppresses AOM/DSS-induced colon tumorigenesis

To determine the roles of CYP monooxygenases in colon cancer, we tested whether genetic ablation of CYP monooxygenases modulates colon tumorigenesis. To this end,

we performed the AOM/DSS-induced colon cancer model in a recently developed *Cyp2c* gene cluster knockout mouse, which has deletions of fourteen mouse *Cyp2c* genes, including *Cyp2c29*, *2c37*, *2c38*, *2c39*, *2c40*, *2c50*, *2c54*, *2c55*, *2c65*, *2c66*, *2c67*, *2c68*, *2c69*, and *2c70* [100] (Figure 4.3A). Compared with $Cyp2c^{+/+}$ mice, the expression of *Cyp2e1* (encoding the enzyme Cyp2e1 to activate the mutagenic activity of AOM [101]) was not changed in $Cyp2c^{+/-}$ or $Cyp2c^{-/-}$ mice, supporting that it is feasible to perform the AOM/DSS-induced colon tumorigenesis model in these mice.

Compared with AOM/DSS-induced $Cyp2c^{+/+}$ mice, the AOM/DSS-induced $Cyp2c^{+/-}$ mice had lower tumor number and total tumor burden (Figure 4.3B), reduced expression of tumorigenic markers proliferating cell nuclear antigen (PCNA) and *β-catenin* (Figure 4.3C), and attenuated expression of pro-inflammatory and pro-tumorigenic genes (*Tnf-α*, *Il-1β, Axin2* and *C-myc*), in the colon tumor tissue (Figure 4.3D)*, illustrating reduced colon tumorigenesis*. In addition, *we found that* compared with AOM/DSS-induced $Cyp2c^{+/+}$ mice, the colon of AOM/DSS-induced $Cyp2c^{+/-}$ mice had lower colonic expression of *Cyp* monooxygenase (using mouse *Cyp2c38* as a marker, Figure 4.3D) and reduced colonic concentrations of CYP monooxygenase-produced EpFAs (Figure 4.3E), supporting the involvement of CYP monooxygenase pathway in colon tumorigenesis.

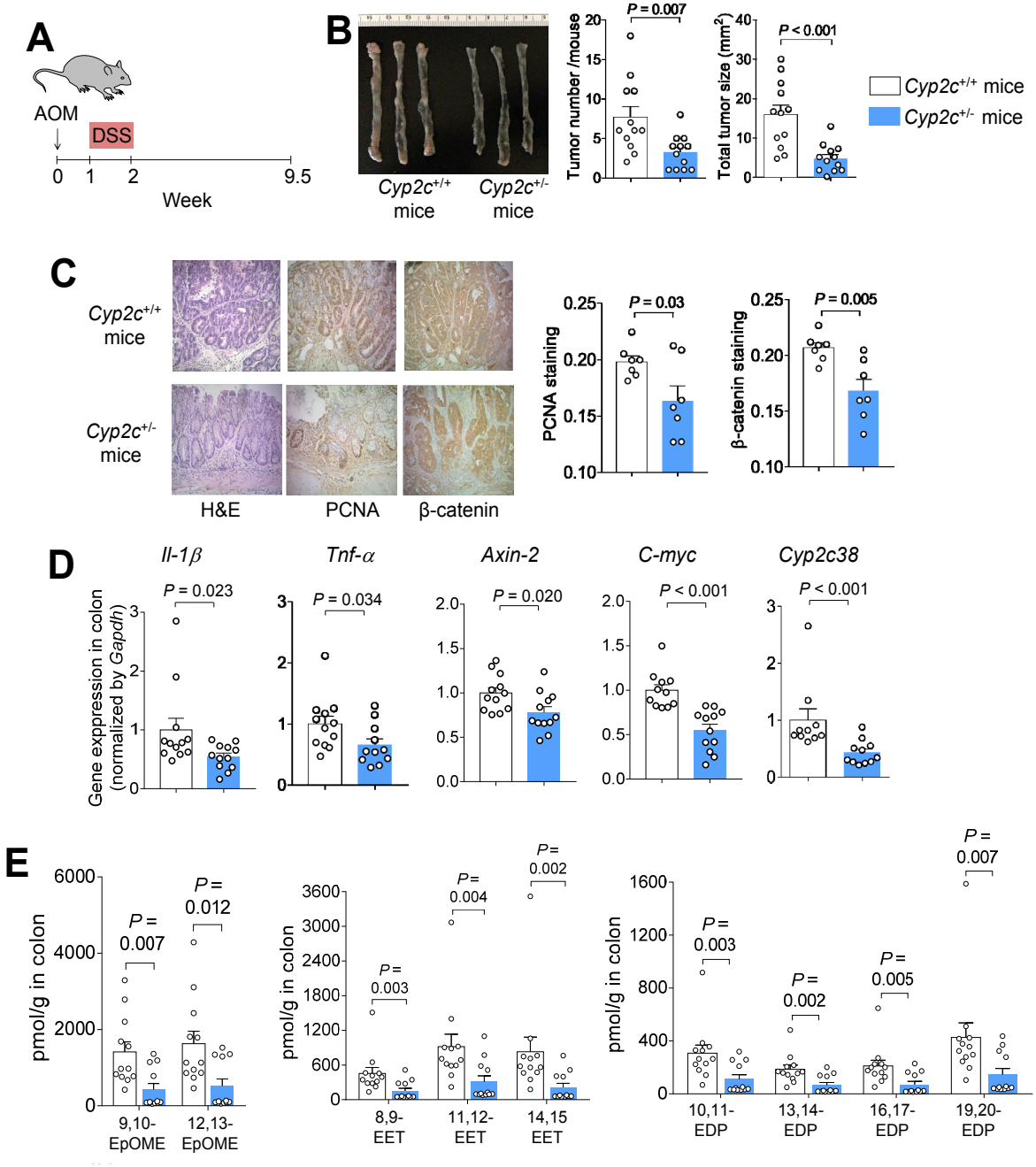

Figure 4.3 Compared with *Cyp2c*$^{+/+}$ mice, the AOM/DSS-induced colon tumorigenesis is reduced in *Cyp2c*$^{+/-}$ mice.

(A) Scheme of animal experiment. (B) Quantification of colon tumorigenesis. (C) H&E histology, and immunohistochemical staining of PCNA and β-catenin in colon. (D) Expression of pro-inflammatory and pro-tumorigenic genes, and Cyp monooxygenase (using Cyp2c38 as a marker) in colon. (E) Concentrations of CYP monooxygenase-produced metabolites in colon.

4.4.5 Pharmacological inhibition of CYP monooxygenases suppresses AOM/DSS-induced colon tumorigenesis

We tested the effects of two different CYP monooxygenase inhibitors, SKF-525A and clotrimazole [102,103], on AOM/DSS-induced colon tumorigenesis in mice. Since the mutagenic activity of AOM requires metabolic activation by Cyp2e1 [101], we initiated treatment of SKF-525A and clotrimazole after the AOM and DSS treatment (see animal experiment scheme in Figure 4.4A). We found that oral administration of these two inhibitors suppressed AOM/DSS-induced colon tumorigenesis in mice (Figure 4.4B). Furthermore, treatment with these two inhibitors reduced expression of PCNA (a marker of cell proliferation), increased expression of cleaved caspase-3 (a marker of cell apoptosis), and decreased expression of pro-inflammatory and pro-tumorigenic genes (*Tnf-α*, *Mcp-1*, *Il-6*, *Il-1β, Ifn-γ*, *Axin2*, and *Cox-2*), in colon tumors (Figure 4.4C-D). These results support that pharmacological inhibition of CYP monooxygenases suppressed AOM/DSS-induced colon tumorigenesis.

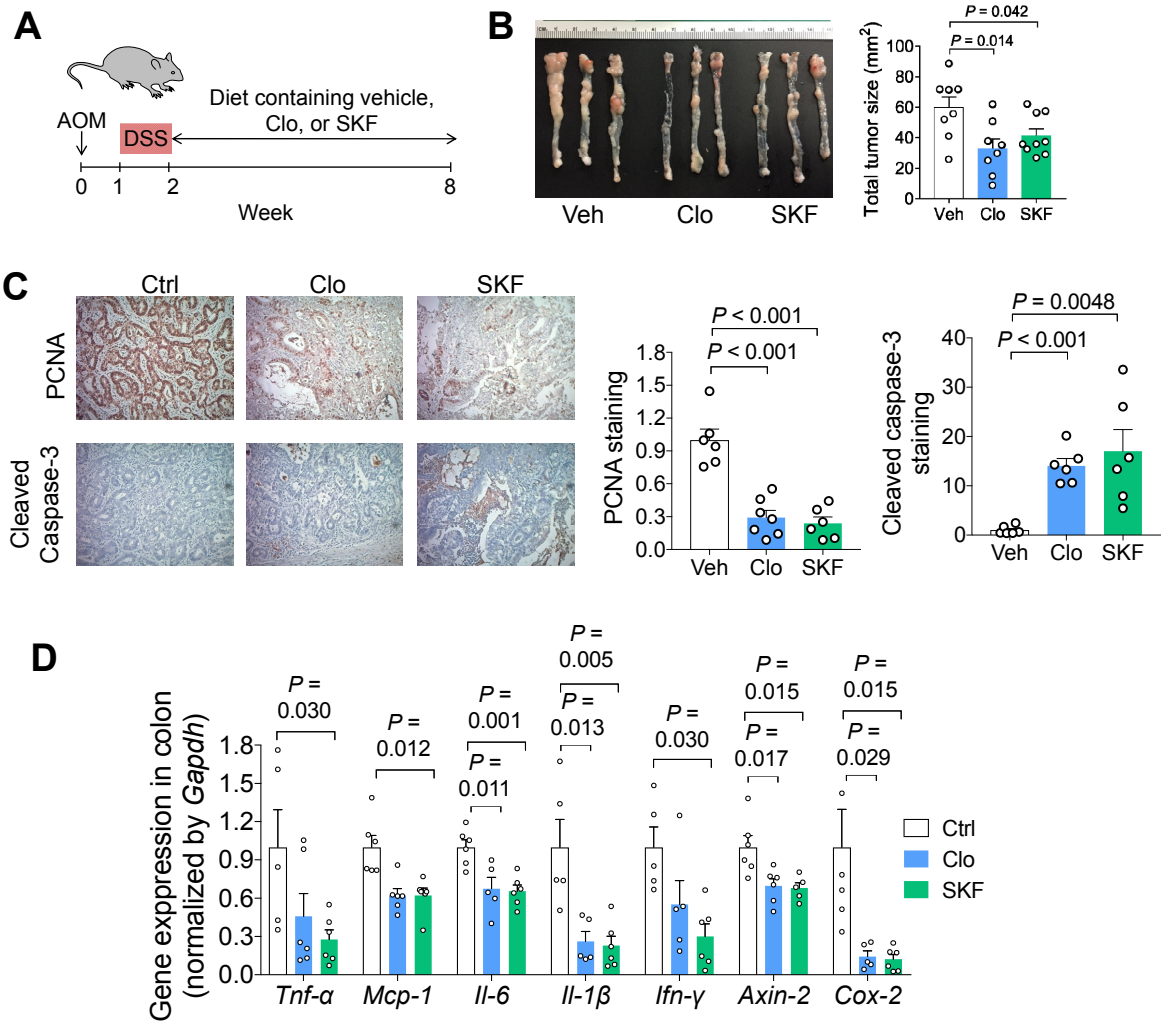

Figure 4.4 Pharmacological inhibition of CYP monooxygenases suppresses AOM/DSS-induced colon tumorigenesis in mice.

(A) Scheme of animal experiment. (B) Quantification of colon tumorigenesis. (C) Immunohistochemical staining of PCNA and cleaved caspase-3 in the colon. (D) Expression of pro-inflammatory and pro-tumorigenic genes in the colon. Abbreviations: Clo: clotrimazole; SKF: SKF-525A.

4.4.6 Treatment with EpOME, but not other CYP monooxygenase metabolites, increases inflammation and JNK phosphorylation in macrophage cells and colon cancer cells

To determine the specific metabolites involved in the colon cancer-enhancing effects of CYP monooxygenases, we studied the biological actions of CYP monooxygenase

metabolites. The ω-6-series CYP metabolites, including EpOMEs produced from LA and EETs produced from ARA, are the most abundant EpFAs in the plasma and tissues, therefore, we focused on these metabolites. Treatment with 9,10- and/or 12,13-EpOME (concentration = 100 nM) increased gene expression of pro-inflammatory cytokines in mouse macrophage RAW 265.7 cells and human colon cancer HCT-116 cells (Figure 4.5A-C). In contrast, other types of CYP metabolites, including the down-stream metabolites of EpOMEs termed 9,10- and 12,13-dihydroxyoctadecenoic acid (DiHOME) or CYP metabolites derived from other fatty acids, such as 11,12- and 14,15-EET, had no such effects (Figure 4.5A-C).

Since 12,13-EpOME showed that most potent effect to induce inflammation *in vitro*, we further studied this metabolite. Treatment with 12,13-EpOME (concentration = 1-100 nM) increased gene expression of *Il-6* and *Mcp-1* in a dose-dependent manner in RAW 265.7 cells (Figure 4.5D). ELISA analysis further validated that 12,13-EpOME increased protein levels of IL-6 and MCP-1 in RAW 265.7 cells (Figure 4.5E). Consistent with its enhancing effect on inflammation, 12,13-EpOME induced a rapid phosphorylation of JNK (Figure 4.5F). Similar results were also observed in colon cancer HCT-116 cells (Figure 4.5G-H). Together, these results demonstrate that 12,13-EpOME had pro-inflammatory effects *in vitro*.

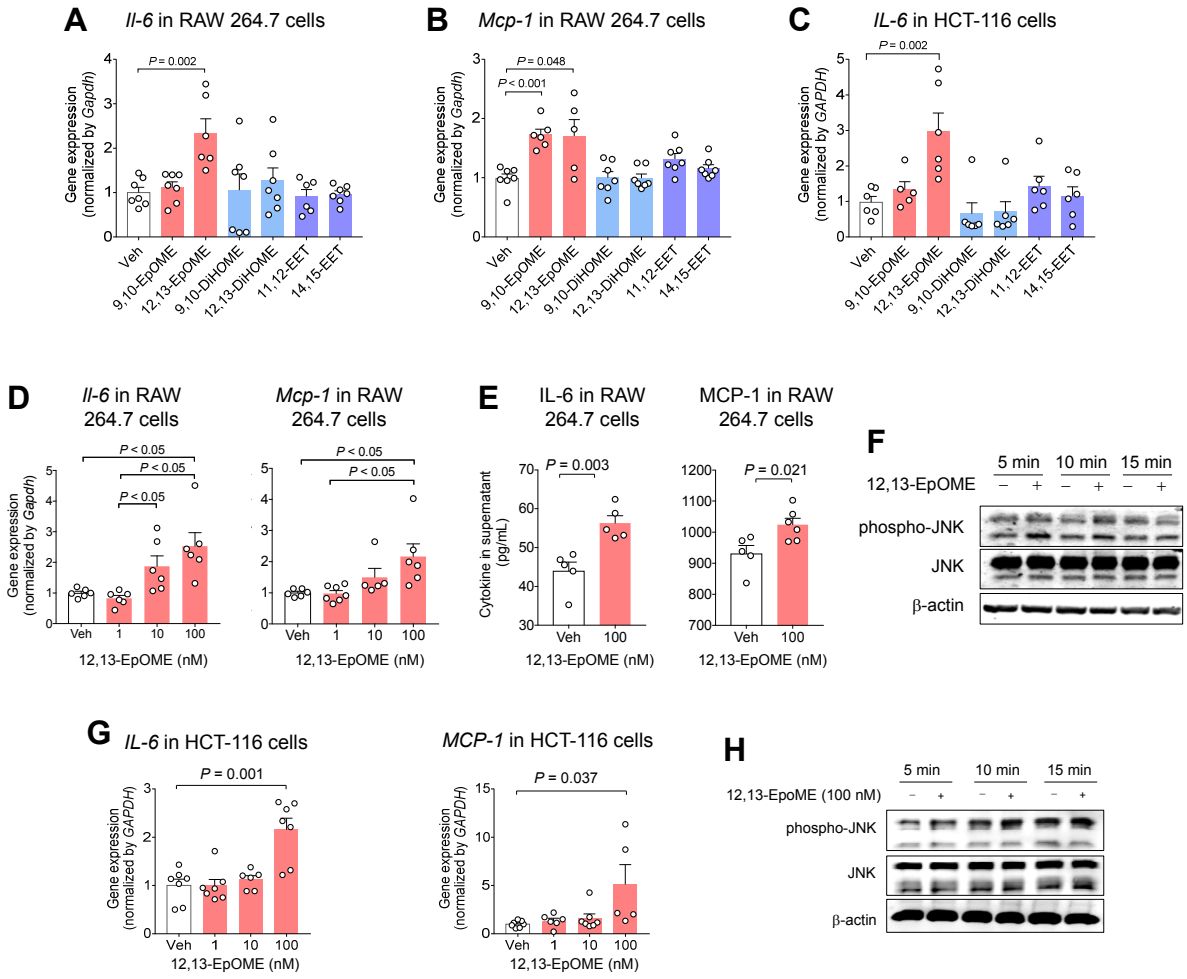

Figure 4.5 EpOME increases inflammation in vitro.
(A-B) Effect of EpOMEs, DiHOMEs and EETs (concentration = 100 nM) on gene expression of Il-6 and Mcp-1 in mouse macrophage RAW 265.7 cells. (C) Effect of EpOMEs, DiHOMEs and EETs (concentration = 100 nM) on gene expression of IL-6 in human colon cancer HCT-116 cells. (D) Dose-response effect of 12,13-EpOME on gene expression of Il-6 and Mcp-1 in RAW 265.7 cells. (E) Effect of 12,13-EpOME on medium concentrations of IL-6 and MCP-1 in RAW 265.7 cells. (F) Effect of 12,13-EpOME on phosphorylation of JNK in RAW 265.7 cells. (G) Dose-response effect of 12,13-EpOME on gene expression of IL-6 and MCP-1 in HC-T116 cells. (H) Effect of 12,13-EpOME on phosphorylation of JNK in HCT116 cells.

4.4.7 Treatment with EpOME exaggerates AOM/DSS-induced colon tumorigenesis *in vivo*

We determined the actions of 12,13-EpOME on colon tumorigenesis *in vivo*. To this end, we stimulated mice with AOM/DSS to induce colon tumors, then treated the mice with

12,13-EpOME or vehicle via Alzet® osmotic mini-pumps (Figure 4.6A). Compared with vehicle-treated AOM/DSS mice, the 12,13-EpOME-treated AOM/DSS mice had increased tumor number, tumor size, and total tumor burden, illustrating exacerbated colon tumorigenesis (Figure 4.6B). Consistent with the increased colon tumorigenesis, treatment with 12,13-EpOME enhanced infiltration of $CD45^+$ and $CD45^+$ $F4/80^+$ immune cells, increased expression of pro-inflammatory and pro-tumorigenic genes (*Tnf-α*, *Il-1β*, and *Axin2*), and upregulated expression of tumorigenic markers (PCNA and *β-catenin),* in the colon tumor (Figure 4.6C-E). Together, these results demonstrate that 12,13-EpOME has enhancing effects on colon tumorigenesis in vivo.

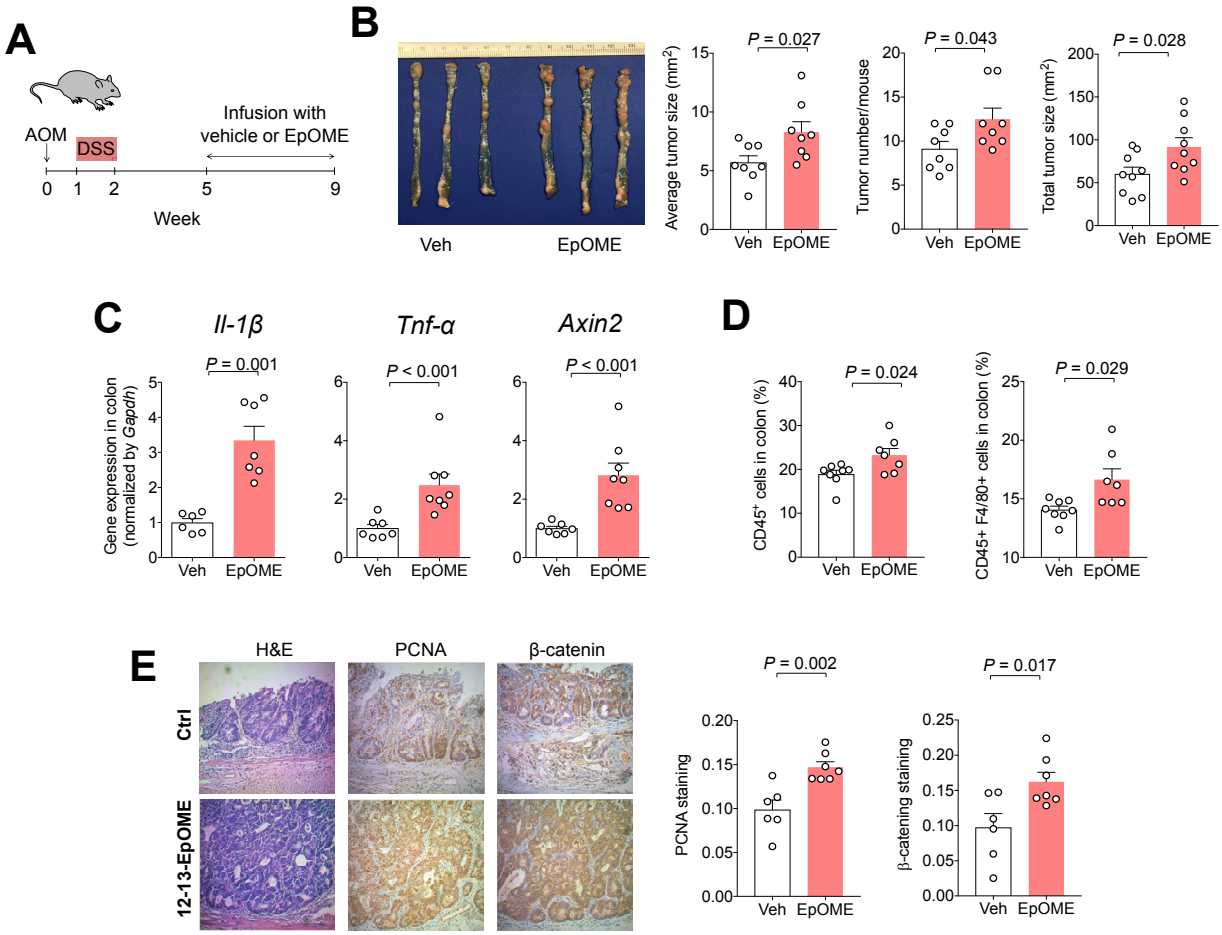

Figure 4.6 EpOME exaggerates AOM/DSS-induced colon tumorigenesis in vivo.

(A) Scheme of animal experiment to test the effect of 12,13-EpOME (dose = 2 mg/kg/day, administered via mini-pump) on colon tumorigenesis. (B) Quantification of colon tumorigenesis in mice. (C) Expression of pro-inflammatory and pro-tumorigenic genes in colon. (D) Quantification of $CD45^+$ and $CD45^+$ $F4/80^+$ immune cells in colon. (E) H&E histology, and immunohistochemical staining of PCNA and β-catenin in colon.

4.5 Discussion

Colon cancer is the third most common cancer and the second leading cause of cancer-related death in United States [6], emphasizing the need for discovery of novel cellular targets which are crucial in the pathogenesis of colon cancer. Using a LC-MS/MS-based targeted metabolomics, the central finding of our research is that EpFAs, which are eicosanoid metabolites produced by CYP monooxygenases, are significantly elevated in both the circulation and colon tissues of the AOM/DSS-induced colon cancer mice. On the basis of this finding, we further demonstrate that CYP monooxygenases are overexpressed in colon cancer and play critical roles in colon tumorigenesis. Together, our findings demonstrate that the previously unappreciated CYP/EpFA axis is upregulated in colon cancer, contributes to its pathogenesis, and could be therapeutically explored for preventing or treating colon cancer.

Here we show that CYP monooxygenase-produced EpFAs are increased in both plasma and colon of the AOM/DSS-induced colon cancer mice. A previous study showed that in DSS-induced colitis models, the circulating concentrations of EpFAs were not changed [36]. Together, these results suggest that colon tumor, but not colonic inflammation, induces the CYP monooxygenase pathway. There could be many mechanisms by which the CYP monooxygenases are overexpressed in colon tumors. The expression of CYP

monooxygenases has been shown to be elevated by hypoxia [3], which is a common feature of tumor tissues [37]. Therefore, the hypoxic tumor microenvironment could contribute to the increased expression of CYP monooxygenases in tumor tissues. To date, the expression pattern of CYP monooxygenases in human colon tumor tissues are not well understood. Only one study has investigated their expressions in human colon tumor tissues, which showed that CYP2C9 is detected in 13 out of 17 human colon tumor samples, while it is not detected in matched benign samples [35]. This result is in agreement with our finding, supporting that the CYP monooxygenase pathway is upregulated in human colon cancer.

Our results support that EpOMEs are critical regulators of colon tumorigenesis. We show that EpOMEs are elevated in the circulation of mice with colon cancer; in addition, EpOMEs have direct and potent effects to induce inflammation and colon tumorigenesis *in vitro* and *in vivo*. A better understanding of the roles of EpOMEs in human colon cancer could help to develop EpOMEs as potential biomarkers of colon cancer, which could have important clinical implications. Previous studies, performed in other disease models, have shown that EpOMEs have an array of detrimental effects on human health. EpOMEs are elevated in the circulation of patients with severe burns, and are associated with multiple organ failure and adult respiratory distress syndrome in these patients [41,42,44,104]. In animal studies, treatment with high-dose EpOMEs induced pulmonary edema, lung injury, and cardio-depression [43,105,106]. Together, these results support that EpOMEs could contribute to the pathogenesis of colon cancer, as well as other human diseases.

The tissue concentrations of EpOMEs are in part mediated by the levels of LA in membrane phospholipids [3]. The consumption of LA, which is highly abundant in vegetable oil products (such as corn, soybean, and canola oils, as well as fried food, salad dressing, and mayonnaise), is very high in western countries [107]. Substantial animal experiments showed that a high dietary intake of LA is associated with increased AOM-induced colon tumorigenesis [62-66], but the underlying mechanisms are not well understood. Here our study showed that EpOMEs have potent effects to induce inflammation and colon tumorigenesis *in vitro* and *in vivo*, suggesting that EpOMEs could serve as a potential mechanistic linkage between overconsumption of LA and elevated risks of colon cancer. Validation of the roles of EpOMEs involved could help to design human studies to clarify the impact of LA consumption on colon tumorigenesis, which could lead to significant impact for public health.

Using both pharmacological and genetic approaches, our results support that CYP monooxygenases could be a potential therapeutic target of colon cancer. Notably, we show that genetic ablation of Cyp2c monooxygenases reduces colonic concentrations of CYP monooxygenase-produced metabolites, and attenuates AOM/DSS-induced colon tumorigenesis. This finding is in agreement with our previous report, which showed that compared with WT mice, genetically engineered mice with endothelial overexpression of CYP2C8 monooxygenase (Tie2-CYP2C8 Tr mice) have enhanced xenograft tumor growth of B16F10 melanoma and T241 fibrosarcoma [28]. Together, these results support that targeting CYP monooxygenases could be a potential strategy to

inhibit colon cancer, as well as other types of cancer. Previous studies showed that some FDA-approved drugs are potent inhibitors of CYP monooxygenases [26], and these drugs could be repurposed for preventing or treating colon cancer; in addition, novel monooxygenase inhibitors could be developed for human translation.

In conclusion, our study demonstrates that the previously unappreciated CYP monooxygenase pathway is upregulated in colon cancer, contributes to its pathogenesis, and could be therapeutically explored for preventing or treating colon cancer. A better understanding of its roles in colon tumorigenesis could help to develop novel therapeutic targets or biomarkers of colon cancer, facilitating the development of mechanism-based strategies to reduce the risks of colon cancer.

CHAPTER 5

Ω-3 POLYUNSATURATED FATTY ACIDS AND THEIR CYTOCHROME P450-DERIVED METABOLITES SUPPRESS COLORECTAL TUMOR DEVELOPMENT IN MICE

5.1 Abstract

Many studies have shown that dietary intake of ω-3 polyunsaturated fatty acids (PUFAs) reduces the risks of colorectal cancer, however the underlying mechanisms are not well understood. Here we used a LC-MS/MS-based lipidomics to explore the roles of eicosanoid signaling in the anti-colorectal cancer effects of ω-3 PUFAs. Our results showed that dietary feeding of ω-3 PUFAs-rich diets suppressed growth of MC38 colorectal tumor, and modulated profiles of fatty acids and eicosanoid metabolites in C57BL/6 mice. Notably, we found that dietary feeding of ω-3 PUFAs significantly increased levels of epoxydocosapentaenoic acids (EDPs, metabolites of ω-3 PUFA produced by cytochrome P450 enzymes) in plasma and tumor tissue of the treated mice. We further showed that systematic treatment with EDPs (dose = 0.5 mg/kg/day) suppressed MC38 tumor growth in mice, with reduced expressions of pro-oncogenic genes such as *c-myc*, *Axin2*, and *C-jun* in tumor tissues. Together, these results support that formation of EDPs might contribute to the anti-colorectal cancer effects of ω-3 PUFAs.

5.2 Introduction

Every year, there are ~134,490 new cases and ~49,190 deaths from colorectal cancer, making colorectal cancer the second cause of cancer-related death in the United States [108]. Epidemiological and pre-clinical data support that dietary intake of ω-3

polyunsaturated fatty acids (PUFAs), which are abundant in fish and fish oil supplements, may reduce risks of colorectal cancer. In contrast, ω-6 PUFAs, which are commonly found in vegetable oils, are suggested to promote colorectal cancer [66,109-113]. This is important because a typical Western diet contains 30-50 times more ω-6 than ω-3 PUFAs, though a ratio of ω-6-to-ω-3 PUFAs of 1:1 was recommended for human consumption by many nutritional panels [114]. Therefore, it is of critical importance to validate the effects and mechanisms of ω-3 PUFAs on colorectal cancer, which might help to effectively implement ω-3 PUFAs for colorectal cancer prevention.

A general mechanism to explain the health benefits of ω-3 PUFAs is that they compete with arachidonic acid (ARA, 20:4ω-6) for the enzymatic metabolism by cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) enzymes, leading to reduced formation of ω-6-series eicosanoids which are predominately pro-inflammatory and pro-tumorigenic, and increased formation of ω-3-series eicosanoid metabolites which are less-detrimental or even beneficial [5]. Recent research showed that ω-3 PUFAs, such as eicosapentaenoic acid (EPA, 20:5ω-3) and docosahexaenoic acid (DHA, 22:6ω-3), are highly efficient alternative substrates of CYP enzymes [4], while they are known as relatively poor substrates of COX and LOX enzymes [115]. Notably, emerging animal and human studies showed that the CYP pathway is the dominant pathway in metabolizing ω-3 PUFAs *in vivo* [116,117]. We showed that epoxydocosapentaenoic acids (EDPs), which are CYP metabolites of DHA, potently suppressed breast tumor growth and lung cancer metastasis through inhibiting angiogenesis *in vivo* [51]. In agreement with our finding, a recent study by Yanai et al. showed that systematic treatment with EDPs inhibited

pathological angiogenesis in a mouse model of macular degeneration [27]. Together, these studies suggest that the CYP metabolites of ω-3 PUFAs, such as EDPs, might play an important role in mediating the anti-cancer and anti-angiogenic effects of ω-3 PUFAs.

Regarding colorectal cancer, previous studies have shown that eicosanoid metabolome is deregulated in colorectal cancer, and some eicosanoid biogenesis enzymes and associated metabolites play central roles in regulating colorectal cancer [19,118]. However, how ω-3 PUFAs modulate eicosanoid signaling in colorectal cancer is poorly characterized, in addition, the actions of ω-3 PUFAs-derived eicosanoid metabolites on colorectal cancer are largely unknown. Here we used a LC-MS/MS-based lipidomics to explore the roles of eicosanoid signaling in the anti-colorectal cancer effects of ω-3 PUFAs, using a xenograft MC38 colorectal cancer model in C57BL/6 mice. Our results showed that dietary feeding of ω-3 PUFA-rich diets suppressed growth of colorectal tumor, and increased levels of EDPs in plasma and tumor tissue of the treated mice. We further found that systematic treatment with EDPs inhibited growth of colorectal cancer in mice. Together, these results support that formation of EDPs could might to the anti-colorectal cancer effects of ω-3 PUFAs.

5.3 Materials and Method

5.3.1 Animal experiment of ω-3 PUFA-rich diet on MC38 colorectal cancer growth

All procedures of animal care were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee of the University of Massachusetts. Male C57BL/6 mice (age = 6 weeks) were purchased from Charles River Laboratories (Wilmington, MA). The mice were pre-fed with experimental diets for three weeks, then

400,000 MC38 colorectal cancer cells (a gift from Prof. Ajit Varki at UCSD) in 100 μL PBS were subcutaneously injected into each mouse to initiate primary tumor growth. Tumor sizes were measured using a caliper; after 15 days of cancer cell injection, the mice were sacrificed and the tumor tissues were dissected, weighted and subjected to biochemical analysis. We used three experimental diets to study the effects of ω-3 PUFAs on colorectal cancer: control ω-6 diet (ratio of ω-6-to-ω-3 PUFA is ≈ 69.3:1), ω-3 diet (ratio of ω-6-to-ω-3 PUFA is ≈ 1.26:1), and ω-3-high diet (ratio of ω-6-to-ω-3 PUFA is ≈ 0.56:1).

5.3.2 Lipidomics analysis

After animal sacrifice, the tumor tissues, colon tissues and plasma were harvested and subjected to LC-MS/MS-based lipidomics analysis. For plasma lipid metabolite extraction, about 250 μL plasma were mixed with deuterated internal standards, then loaded onto pre-washed Waters® Oasis solid phase extraction (SPE) cartridges, washed with 95:5 water/methanol with 0.1% acetic acid, the analytes were eluted with methanol and ethyl acetate, dried using a centrifugal vacuum evaporator, then reconstituted in methanol for LC-MS/MS analysis. For tumor lipid metabolite extraction, about 100 mg tissues were mixed with an antioxidant solution (0.2 mg/mL butylated hydroxytoluene and 0.2 mg/mL triphenylphosphine in methanol), the deuterated internal standards, and 400 μL extract solution (0.1% acetic acid with 0.2 mg/mL butylated hydroxytoluene in a methanol solution), were homogenized; the resulting homogenates were kept in −80 °C overnight. After centrifugation of the homogenates, the pellets were washed with methanol (containing 0.1% butylated hydroxytoluene and 0.1% acetic acid) and then

combined with the supernatant. The lipid metabolites in the combined solutions were extracted using SPE columns, similar to the description above for the plasma lipid metabolite extraction. The LC-MS/MS analysis were carried out on an Agilent 1200SL HPLC system (Agilent, Santa Clara, CA) coupled to a 4000 QTRAP MS/MS (AB Sciex, Foster City, CA) as described in our previous report 18. The peaks were identified according to the retention time and specific multiple reaction monitoring (MRM) transitions of the lipid metabolite standards. The concentrations of the lipid metabolites are calculated against the calibration curve with standards.

5.3.3 Flow cytometry analysis

After animal sacrifice, the dissected tumor tissues were digested using enzymatic degradation solution (500 μg/ml collagenase, 500 μg/ml DNase, 100 μg/ml Hyaluronidase in HBSS) for 2 hours at room temperature, filtered through 70 μm cell sorters (BD Biosciences) to obtain single cell suspension, which were stained with FITC-conjugated anti-mouse CD45 antibody, APC-conjugated anti-mouse CD31 antibody, APC/Cy7 anti-mouse Ly-6G/Ly-6C (Gr-1) antibody, PerCP/Cy6.5-conjugated anti-mouse F4/80 antibody or isotype control antibody (R&D Systems). The stained cells were analyzed using BD LSRFortessa™ cell analyzer (BD Biosciences) and data were processed using FlowJo software. Endothelial cells were identified as CD45-, CD31+ cells, neutrophils were identified as CD45+, Gr1+ cells and macrophage were identified as CD45+, F4/80+ cells.

5.3.4 Animal experiment of EDPs on MC38 colorectal cancer growth

To test the effect of EDPs on colorectal cancer growth, C57BL/6 male mice were subcutaneously implanted with Osmotic mini-pumps (Durect, Cupertino, CA, catalog number 1004), which contained vehicle (a 1:1 vol/vol mixed solution of DMSO and PEG 400) or EDPs (dose of EDPs = 0.5 mg/kg body weight/day). After one week of mini-pump implantation, 400,000 MC38 colorectal cancer cells in 100 μL PBS were subcutaneously injected into each mouse to initiate primary tumor growth. Tumor sizing was measured using caliper, at the end of the experiment, the tumors were dissected, weighted and subjected to biochemical analysis. The EDPs are a mixture containing EDP regioisomers (7,8-, 10,11-, 13,14-, 16,17-, and 19,20-EDP), as we described 19.

5.3.5 Real-time PCR (RT-PCR) analysis

Total RNA was isolated from tumor tissues using TRIzol Reagent (Life technologies, Carlsbad, CA) according to manufacturer's instruction. Conversion of up to 2 μg of total RNA to single stranded cDNA was preformed using High-Capacity cDNA Reverse Transcription Kit (Life technologies, Carlsbad, CA) according to manufacturer's instruction. Quantitative RT-PCR was conducted using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Agawam, MA) on a DNA Engine Opticon® 2 System (Bio-Rad Laboratories, Hercules, CA) with specific mouse primers. The primers used in this research were: C-myc (sense) 5'-ATGCCCCTCAACGTGAACTTC-3' and (antisense) 5'-GTCGCAGATGAAATAGGGCTG-3',

Axin2 (sense) 5'-TGACTCTCCTTCCAGATCCCA-3' and (antisense) 5'-TGCCCACACTAGGCTGACA-3', C-jun (sense) 5'-CCTTCTACGACGATGCCCTC-3' and (antisense) 5'-GGTTCAAGGTCATGCTCTGTTT-3', The results of target genes were normalized to Gapdh gene and expressed to the control group mice using the 2-ΔΔCt method.

The primer to analyze Gapdh is (sense) 5'-AGGTCGGTGTGAACGGATTTG-3' and (antisense) 5'-TGTAGACCATGTAGTTGAGGTCA-3'.

5.3.6 Data Analysis

All data are expressed as the mean ± standard error of the mean (SEM). Differences among Control, DHASCO and DHASCO-high diet feeding group were analyzed by one-way ANOVA and pairwise multiple comparisons among groups were performed by using Dunn's test. For the comparison between the control group and EDP group, Shapiro-Wilk test was used to verify the normality of data. When data were normally distributed, statistical significance was determined using two-side t-test; otherwise, significance was determined by Mann-Whitney U test. P values less than 0.05 are reported as statistically significant. All of these data analysis was performed by using SigmaPlot software (San Jose, CA).

5.4 Results

5.4.1 ω-3 PUFAs-rich diets inhibit growth of MC38 colorectal tumor in mice

We prepared three completely defined isocaloric diets containing 10 wt/wt% total fat. For the control diet, corn oil was the only source of dietary fat, with a ratio of ω-6-to-ω-3 PUFAs in the diet = 69.3:1, to mimic a typical Western diet. For the DHASCO diet, the

fat component was a mixture of 38% corn oil and 62% DHA-rich DHASCO® Algae oil, with a ratio of ω-6-to-ω-3 PUFAs = 1.26:1. For the DHASCO-high diet, the fat component was a mixture of 20.7% corn oil and 79.3% DHASCO® oil, with a ratio of ω-6-to-ω-3 PUFAs = 0.56:1. We have designed these two ω-3 PUFAs-rich diets, since a ratio of ω-6-to-ω-3 PUFAs ≈ 1:1 was recommended for human consumption by nutritionists [119].

To study the effect of ω-3 PUFAs on MC38 colorectal tumor growth, we pre-fed 6-week-old C57BL/6 mice with these three experimental diets for three weeks, then subcutaneously injected MC38 colorectal cancer cells into mice to initiate growth of colorectal tumor (see animal experimental scheme in Figure 5.1A). Compared with control diet, both DHASCO and DHASCO-high diets suppressed growth of MC38 colorectal tumor in mice with similar inhibitory effects: at the end of the experiment, these two ω-3 PUFAs-rich diets caused a ~50% reduction of tumor weight ($P < 0.05$, Figure 5.1B-D). Flow cytometry analysis of the single cell suspension from the dissected MC38 tumors showed that these two ω-3 PUFAs-rich diets reduced the presence of endothelial cells (CD31+, CD45-) in tumor tissues, suggesting that dietary feeding of ω-3 PUFAs suppressed tumor angiogenesis ($P < 0.05$, Figure 5.1E). Together, these results are in agreement with previous studies for the anti-cancer and anti-angiogenic effects of ω-3 PUFAs.

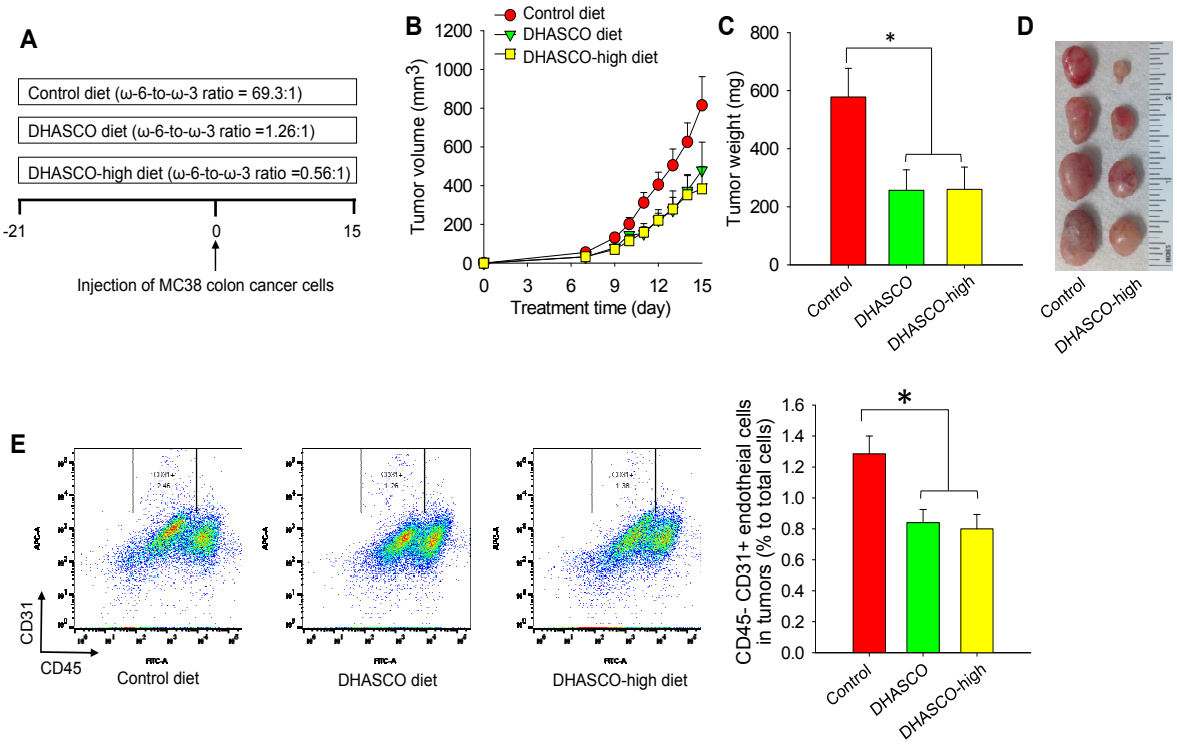

Figure 5.1 Dietary feeding of ω-3 PUFAs-rich diets suppressed growth of MC38 colorectal tumor, and tumor angiogenesis in C57BL/6 mice.
(A) scheme of animal experiment, (B) time-course of MC38 tumor sizing, (C) quantification of tumor weight, (D) representative image of dissected MC38 tumors, (E) flow cytometry quantification of endothelial cells in MC38 tumors.

5.4.2 ω-3 PUFAs-rich diets modulated fatty acid profiles in MC38 colorectal tumors

We used GC-MS to analyze the profiles of fatty acids in the dissected MC38 colorectal tumors. Dietary feeding of the DHASCO diet (ratio of ω-6-to-ω-3 PUFAs = 1.26:1) significantly reduced levels of ω-6 PUFAs such as ARA, and increased levels of ω-3 PUFAs such as EPA and DHA in MC38 tumor tissues. Notably, the level of ARA in MC38 colorectal tumor tissues was reduced from 9.96 ± 1.71% (mean ± SEM, from mice fed on control diet, the result was expressed as % of ARA to the total fatty acids in MC38 tumor tissues) to 2.89 ± 0.38% (from mice fed on DHASCO diet, $P < 0.01$), representing a ~70% reduction of ARA in tumor tissues. The level of EPA was increased from 0 ± 0 %

to 1.27 ± 0.19% ($P < 0.001$), and the level of DHA was increased from 0.91 ± 0.16% to 8.33 ± 1% ($P < 0.001$). In contrast, the levels of other PUFAs, such as linoleic acid (LA, 18:2ω-6) and α-linoleic acid (ALA, 18:3ω-3), were not significantly changed in the tumor tissues. Together, these results showed that dietary feeding of ω-3 PUFAs-rich diet modulated profiles of PUFAs in MC38 colorectal tumor tissues, with reduced ARA and increased EPA and DHA.

5.4.3 ω-3 PUFAs-rich diets modulated profiles of eicosanoid metabolites in plasma and tumor tissues

After demonstrating that dietary feeding of ω-3 PUFAs-rich diets modulated tissue profiles of ARA, EPA, and DHA, which are important precursors to generate eicosanoid metabolites [5], we studied the impacts of ω-3 PUFAs-rich diets on profiles of eicosanoid metabolites in the treated mice. To this end, we used a LC-MS/MS-based lipidomics approach to analyze the profiles of eicosanoid metabolites extracted from plasma, MC38 tumor, and colon tissues of the treated mice. Our LC-MS/MS method can analyze > 100 eicosanoid metabolites (see complete list of the eicosanoid metabolites analyzed by our method in Table 3.1).

The LC-MS/MS analysis successfully quantified 58, 44, and 44 eicosanoid metabolites in plasma, MC38 tumor, and colon tissues, respectively; and some metabolites were below the detection limits of our LC-MS/MS method. The detected compounds included eicosanoid metabolites produced by COX, LOX, and CYP enzymes from LA, ALA, ARA, EPA, and DHA [86], allowing us to systematically analyze the effects of ω-3 PUFAs

on eicosanoid metabolome. A general trend was that dietary feeding of ω-3 PUFAs-rich diets reduced levels of ω-6-series metabolites, and increased levels of ω-3-series metabolites in plasma and tissues, which is in agreement with previous studies [5]. The details of the LC-MS/MS profiling are described below.

The CYP epoxygenases (mainly CYP2C and CYP2J isoforms) convert ARA, EPA, and DHA to fatty acid epoxides termed epoxyeicosatrienoic acids (EETs), epoxyeicosatetraenoic acids (EEQs), and EDPs respectively, which are further metabolized by soluble epoxide hydrolase (sEH) to generate the corresponding fatty acid diols (see scheme of CYP pathway in Figure 5.2A) [3]. In the mice fed with control diet (ω-6 PUFAs-rich), ARA-derived EETs are among the most abundant fatty acid epoxides in plasma (Figure 5.2B). In contrast, in the plasma of mice fed with DHASCO and DHASCO-high diets, there was a dramatic reduction of EETs and an increase of EPA-derived EEQs and DHA-derived EDPs; notably, DHA-derived EDPs were among the most abundant fatty acid epoxides in plasma (Figure 5.2B). Consistent with the trend of fatty acid epoxides, dietary feeding of ω-3 PUFAs-rich diets also reduced ARA-derived fatty acid diols termed dihydroxyeicosatrienoic acids (DHETs), and enhanced EPA-derived dihydroxyeicosatetraenoic acids (DiHETEs) and DHA-derived dihydroxydocosapentaenoic acids (DiHDPEs) (see Figure 5.2B). Similar to the profiles of eicosanoid metabolites in plasma, we also found that dietary feeding of ω-3 PUFAs-rich diets reduced levels of ARA-derived fatty acid epoxides and diols, and increased levels of EPA- and DHA-derived epoxides and diols in MC38 tumor and colon tissues (Figure 5.2C-D).

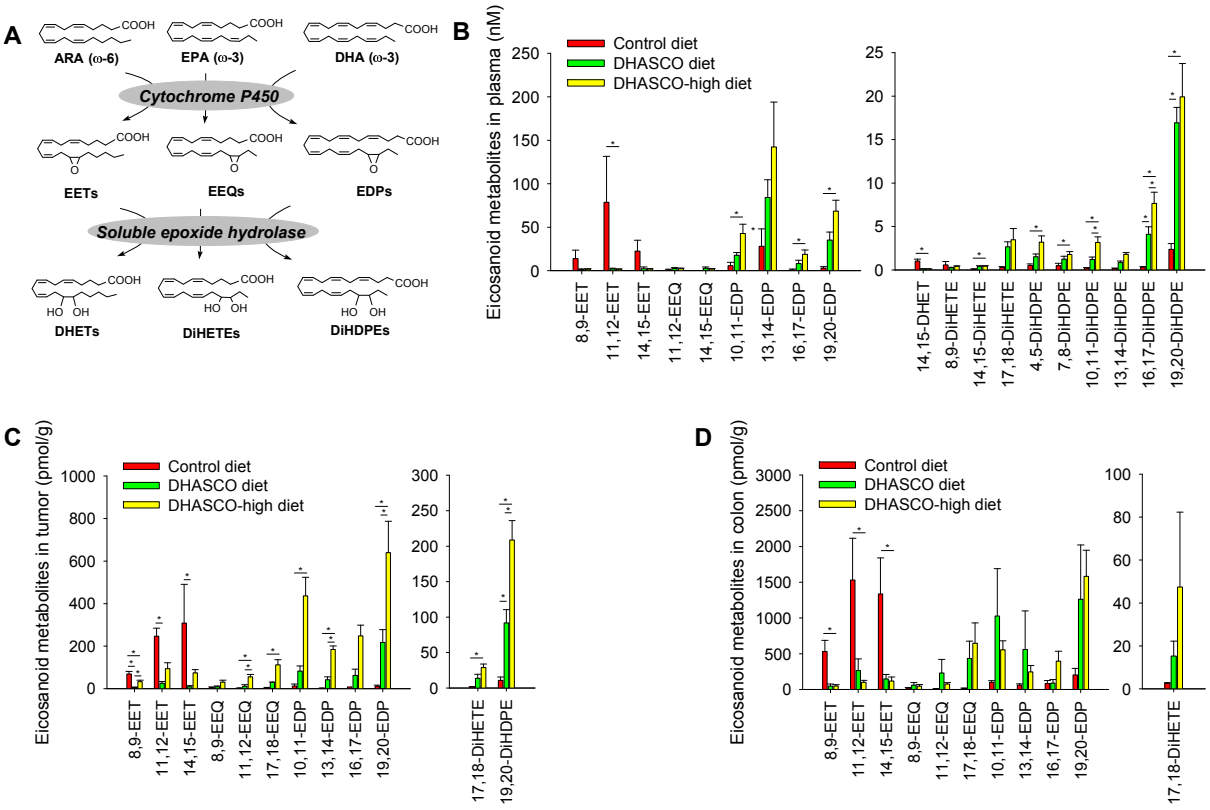

Figure 5.2 Dietary feeding of ω-3 PUFAs-rich diets modulated profiles of eicosanoid metabolites in mice.

(A) scheme of CYP pathway. (B) plasma, (C) MC38 tumor, and (D) colon profiles of CYP-derived fatty acid epoxide and diols.

Besides the CYP pathway, we also analyzed eicosanoid metabolites produced by COX and LOX enzymes, many of these metabolites play central roles in regulating inflammation and tumorigenesis. For COX pathway, dietary feeding of ω-3 PUFAs-rich diets reduced levels of ARA-derived prostaglandins, notably, ω-3 PUFAs caused ~80% reduction of prostaglandin E_2 (PGE_2), which has potent pro-inflammatory and pro-tumorigenic actions, in both MC38 tumor and colon tissues (Figure 5.3A-C). For LOX pathway, dietary feeding of ω-3 PUFAs-rich diets reduced ARA-derived hydroxyl fatty acids such as 11- and 15-hydroxyeicosatetraenoic acid (HETE), and increased EPA-derived 5- and 15-hydroxyeicosapentaenoic acid (HEPE) (see Figure 5.3D-F). Together,

these results showed that dietary feeding of ω-3 PUFAs-rich diets caused a profound change of the eicosanoid metabolome, with reduced levels of ARA-derived metabolites, and increased levels of EPA- and DHA-derived metabolites.

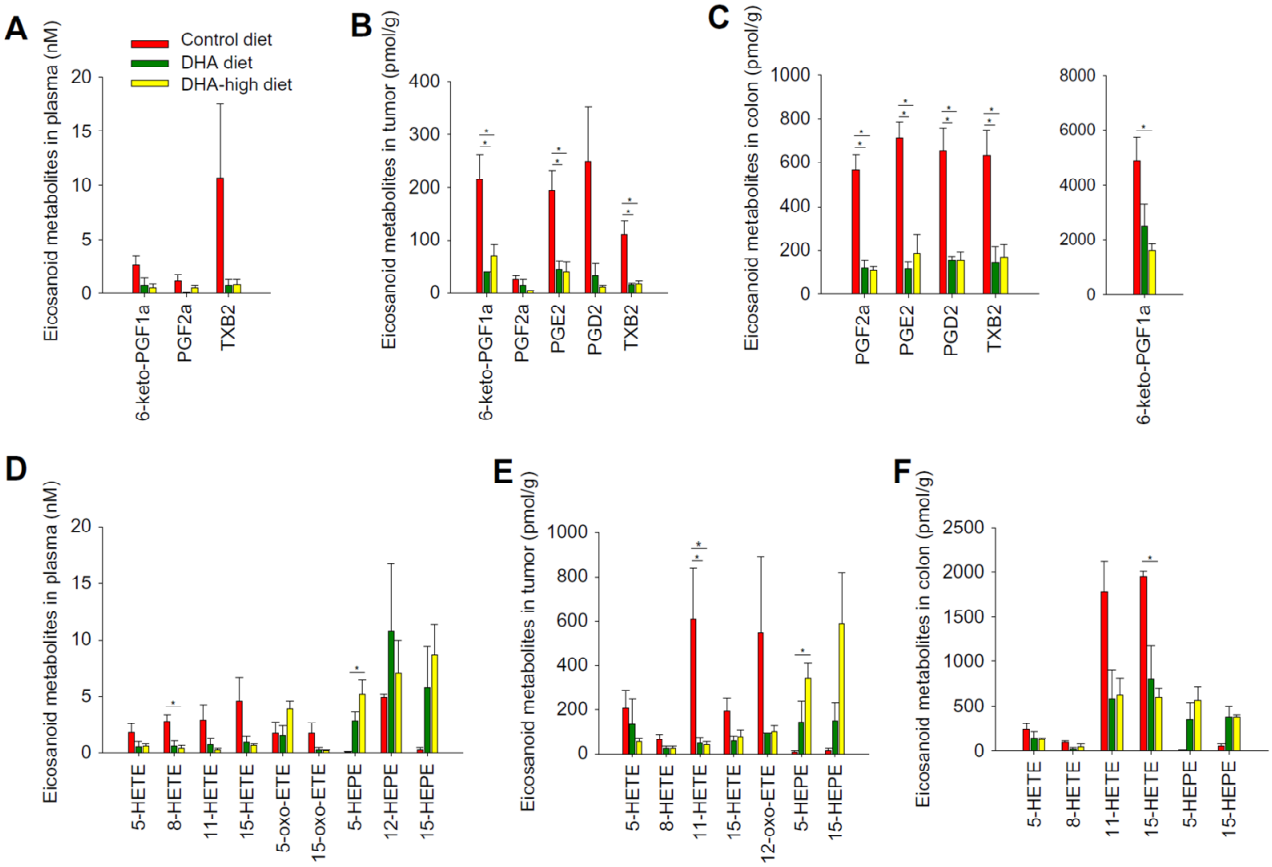

Figure 5.3 Dietary feeding of ω-3 PUFAs-rich diets modulated profiles of eicosanoid metabolites from COX and LOX pathway in mice.
(A) Plasma, (B) MC38 tumor, and (C) Colon profiles of COX-derived prostaglandins and thromboxanes. (D) plasma, (E) MC38 tumor, and (F) colon profiles of LOX-derived metabolites.

5.4.4 EDPs suppressed growth of MC38 tumor growth *in vivo*

Given that dietary feeding of ω-3 PUFAs-rich diets dramatically increased concentrations of EDPs in plasma and tissues, we tested the effect of EDPs on MC38 tumor growth in C57BL/6 mice. Continuous infusion of synthetic EDPs (dose = 0.5 mg/kg/day) inhibited MC38 tumor growth in C57BL/6 mice, with ~50% reduction of tumor weight (Figure

5.4A-B). Flow cytometry of the single cell suspension from the dissected MC38 tumors showed that EDPs slightly reduced infiltration of neutrophils and macrophages into tumor tissues ($P < 0.05$, Figure 5.4C). RT-PCR analysis showed that EDPs reduced expressions of several genes related to cellular kinetics and tumorigenic Wnt pathway, such as *c-myc*, *Axin2*, and *C-jun* (Figure 5.4D), which are consistent with the anti-tumor effect of EDPs.

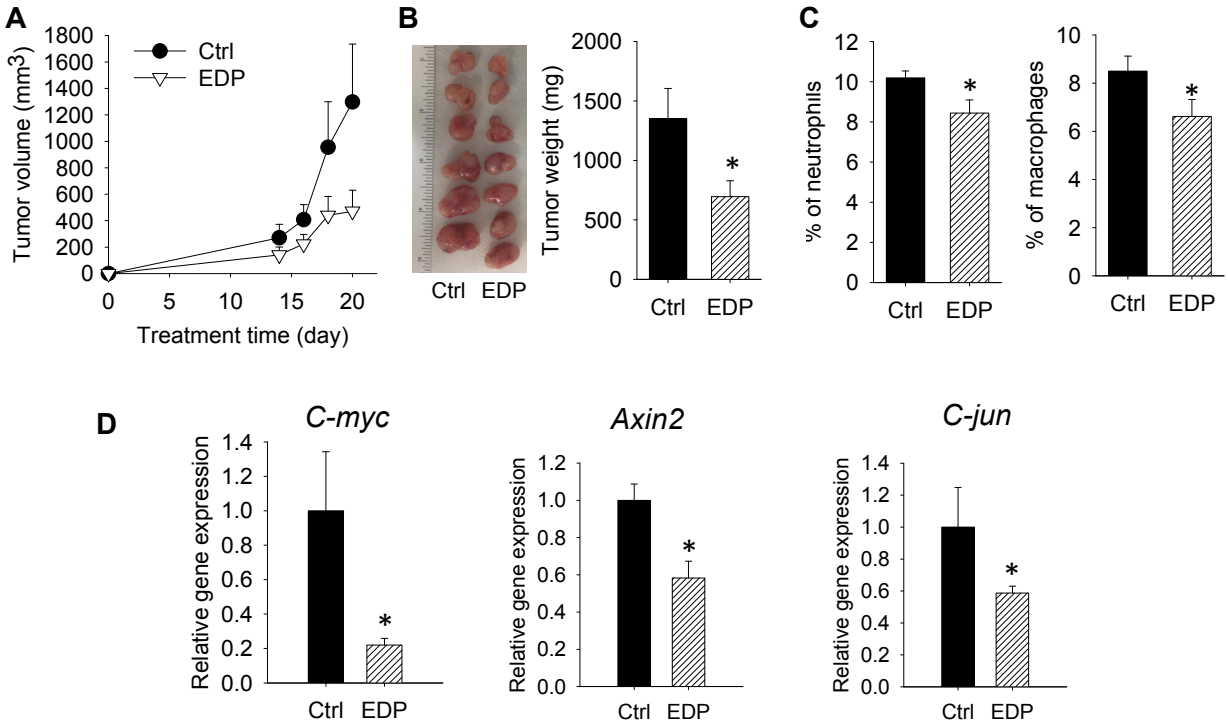

Figure 5.4 Treatment with synthetic EDPs suppressed growth of MC38 tumor in C57BL/6 mice.

(A) time-course of MC38 tumor sizing, (B) representative images of dissected MC38 tumors, and quantification of tumor weight, (C) flow cytometry analysis of immune cells in MC38 colon tumors, and (D) RT-PCR analysis of pro-oncogenic genes in MC38 tumors.

5.5 Discussion

To date, there is a fair amount of studies to demonstrate the anti-colorectal cancer effects of ω-3 PUFAs [66,109-113]; however, the underlying mechanisms remain largely unknown. A

general theory to explain the health benefits of EPA and DHA is that they can efficiently compete with ARA at almost every step of eicosanoid biogenesis, leading to reduced formation of ARA-derived eicosanoids which are predominately pro-inflammatory and/or pro-tumorigenic, and increased formation of EPA- and DHA-derived metabolites which have less-detrimental or beneficial actions [5]. Here we used a LC-MS/MS-based lipidomics to explore the roles of eicosanoid signaling in the anti-colorectal cancer effects of ω-3 PUFAs. The LC-MS/MS analysis showed that dietary feeding of ω-3 PUFA-rich diets caused a dramatic modulation of the eicosanoid profiles in circulation and in colorectal tumor tissues, with reduced levels of ω-6-series metabolites and increased levels of ω-3-series metabolites. A central finding of our study is that dietary feeding of ω-3 PUFAs-rich diets dramatically increased levels of EDPs in both plasma and MC38 colorectal tumor of the treated mice. In addition, systematic treatment with EDPs suppressed growth of MC38 colorectal tumor in mice. Together, these results support that formation of EDPs could contribute the anti-colorectal cancer effects of ω-3 PUFAs, and EDPs might serve as a biomarker for the anti-cancer effects of ω-3 PUFAs.

Regarding the biological effects of EDPs, our results further support the anti-cancer effects of EDPs. Our previous study showed that stabilized EDPs suppressed breast tumor growth and Lewis lung carcinoma metastasis in mice [51]. However, in our previous study, co-administration of a pharmacological inhibitor of soluble epoxide hydrolase (sEH, the major enzyme in degrading EDPs) was required to stabilize low-dose EDP (dose of EDP = 0.05 mg/kg/day in our previous study) in circulation, in order to demonstrate the anti-cancer and anti-metastatic effects of EDP *in vivo* [51]. Based on our

previous study, here we hypothesize that administration of EDP alone, at a higher dose, inhibits tumor growth in mice. Indeed, our result showed that administration of EDPs alone, at a higher dose than that of our previous experiment (dose of EDPs in this study = 0.5 mg/kg/day), significantly inhibited MC38 colorectal tumor growth in mice, further validating the anti-cancer effects of EDPs *in vivo*. Several animal and human studies have shown high levels of EDPs in circulation upon dietary intake of ω-3 PUFAs [116,117], supporting that EDPs could serve as a reliable biomarker for exposure to ω-3 PUFAs. A limitation of current study is that we only tested the effects of exogenously administered EDPs, the biological effects of endogenous EDPs remain largely unknown. Further studies are needed to better characterize the actions of EDPs, in order to facilitate the development of potential biomarkers of ω-3 PUFAs.

Many studies support that ω-3 PUFAs reduce the risks of colon inflammation [120-122] and colon cancer [66,109-113]. However, there are inconsistent results from animal and human studies, which showed that ω-3 PUFAs had no effect [123,124] or detrimental effects [125,126], making it difficult to implement ω-3 PUFAs for disease prevention. The mixed results for the health-promoting effects of ω-3 PUFAs is a major barrier to effectively implement ω-3 PUFAs for disease prevention. Based on our studies, polymorphisms in the genes encoding enzymes in CYP pathway may affect the metabolism of ω-3 PUFAs [127-133], impacting the generation of bioactive lipid metabolites, and thereby contributing to observed inter-individual variations to ω-3 PUFA supplementation [134]. For example, based on our findings, people carrying Lys55Arg and Cys154Tyr mutations of soluble epoxide hydrolase (sEH, the major enzyme to degrade EDPs), which lead to higher sEH

enzymatic activities [133], might have lower tissue levels of EDPs, and thus poorer anti-cancer responses upon dietary ω-3 PUFA supplementation. Such mechanistic knowledge, together with utilization of nutrigenomic and metabolomic approaches, could lead to targeted human trials to better understand the metabolic individuality and nutrition effects of ω-3 PUFAs [134,135].

In conclusion, our studies showed that dietary feeding of ω-3 PUFAs-rich diets dramatically modulated profiles of eicosanoid metabolites in a mouse model of colorectal cancer. Among the altered eicosanoid metabolites, DHA-derived EDPs could play critical roles in mediating the anti-colorectal cancer effects of ω-3 PUFAs. A limitation of our study is that we used a xenograft model of MC38 colorectal cancer, which has many limitations to mimic the complicated process of colorectal carcinogenesis. In addition, we only studied the biological effects of exogenously administered EDPs. Further studies are needed to better characterize the roles of EDPs in health benefits of ω-3 PUFAs, in order to establish potential biomarkers for effective implementation of ω-3 PUFAs.

CHAPTER 6

SUMMARY

Enzymatic metabolism of polyunsaturated fatty acids leads to formation of bioactive LMs. Previous studies have shown that obesity leads to deregulation of LMs in adipose tissues. However, most previous studies have focused on single or limited number of LMs, few systematical analyses have been carried out. In **Research Project 1**, we conducted a LC-MS/MS-based lipidomics analysis of HFD-induced obesity in mice. Our central finding in **Research Project 1** is that HFD significantly modulated the profiles of LMs in adipose tissues of mice. Among the three major PUFA metabolizing pathways (COX, LOX and CYP), CYP-derived fatty acid epoxides are the most dramatically changed LMs in adipose tissues of HFD-induced obesity. Almost all types of fatty acid epoxides, including EpOMEs derived from LA, EETs from ARA, EpDPEs from DHA, and EpODEs from α-LA, were reduced by 70-90% in different types of adipose tissues. Consistent with the reduced levels of fatty acid epoxides, the gene expressions of several CYP monooxygenases, including *Cyp2j5, Cyp2j6,* and *Cyp2c44,* were significantly reduced in adipose tissues of high-fat diet-fed mice. Our results showed that CYP-derived fatty acid epoxides are the most responsive LMs in high-fat diet-induced obesity, suggesting that these novel LMs could play critical roles in pathology of obesity. This study lays the foundation to further investigate the functional roles of LMs in obesity, which could facilitate the development of novel biomarkers or therapeutic targets for obesity and obesity-associated diseases.

Colon cancer is the third most common cancer and the second leading cause of cancer-related death in the United States. It is important to discover novel cellular targets which are crucial in the pathogenesis of colon cancer, which could facilitate development of mechanism-based strategies to reduce the risks of colon cancer. Previous research has shown that certain eicosanoid metabolites are deregulated in colon cancer and contribute to its pathogenesis. Notably, the most prominent cancer-associated eicosanoids are prostaglandins, which are produced by COX-2 that is overexpressed in most human colon cancer samples. However, the gastrointestinal and cardiovascular toxicities induced by COX-2 inhibitors have limited their clinical applications [22]. Besides COX-2, the roles of other eicosanoid pathways in colon tumorigenesis are not well understood. In **Research Project 2**, we used a lipidomics approach to systematically profile eicosanoids in a well-established AOM/DSS-induced colon cancer model in C57BL/6 mice, then use pharmacological and genetic approaches to validate the functional roles of identified metabolite in colon tumorigenesis. Our central finding in **Research Project 2** is that EpFAs, which are eicosanoid metabolites produced by CYP monooxygenases, are significantly elevated in both the circulation and colon tissues of the AOM/DSS-induced colon cancer mice. On the basis of this finding, we further demonstrate that CYP monooxygenases are overexpressed in colon cancer and play critical roles in colon tumorigenesis. Together, our findings demonstrate that the previously unappreciated CYP/EpFA axis is upregulated in colon cancer, contributes to its pathogenesis, and could be therapeutically explored for preventing or treating colon cancer.

Regarding colorectal cancer, previous studies and Specific Aim 2 have shown that eicosanoid metabolome is deregulated in colorectal cancer, and some eicosanoid

biogenesis enzymes and associated metabolites play central roles in regulating colorectal cancer [19,118]. However, how ω-3 PUFAs modulate eicosanoid signaling in colorectal cancer is poorly characterized, in addition, the actions of ω-3 PUFAs-derived eicosanoid metabolites on colorectal cancer are largely unknown. In **Research Project 3**, we used a lipidomics approach to explore the roles of eicosanoid signaling in the anti-colorectal cancer effects of ω-3 PUFAs, using a xenograft MC38 colorectal cancer model in C57BL/6 mice. Our central finding in **Research Project 3** is that dietary feeding of ω-3 PUFAs-rich diets dramatically increased levels of EDPs in both plasma and MC38 colorectal tumor of the treated mice. In addition, systematic treatment with EDPs suppressed growth of MC38 colorectal tumor in mice. Together, these results support that formation of EDPs could contribute the anti-colorectal cancer effects of ω-3 PUFAs, and EDPs might serve as a biomarker for the anti-cancer effects of ω-3 PUFAs.

APPENDIX A

PUBLICATIONS

1. **Wang W***, Yang J.*, Zhang J.*, Wang Y., Hwang S., Qi W., Wan D., Kim, D., Sun, J., Sanidad K., Yang H., Park Y., Liu, J.-Y., Zhao, X., Zheng, X., Liu, Z., Hammock, B. D., & Zhang, G.: Lipidomic profiling reveals soluble epoxide hydrolase as a therapeutic target of obesity-induced colonic inflammation. Proc. Natl. Acad. Sci. U.S.A. 2018, 115(20):5283-5288.
(Media coverage by Boston Globe, Science Daily, AAAS-EurekAlert! Science News, News & Media of UMass Amherst and UC Davis)

2. Yang H*., **Wang W***., Romano K.A., Gu M., Sanidad K., Kim D., Yang J., Schmidt B., Panigrahy D., Pei R., Martin D.A., Ozay E.I., Wang Y., Song M., Bolling B.W., Xiao H., Minter L.M., Yang G., Liu Z., Rey F.E., and Zhang G.: A common antimicrobial additive increases colonic inflammation and colitis-associated colon tumorigenesis in mice. Science Translational Medicine 2018, 10(443): eaan4116.
(Media coverage by U.S. News & World Report, Los Angeles Times, Canadian Broadcasting Corporation (CBC))

3. **Wang W.***, Yang J.*, Edin M.*, Wang Y.*, Luo Y., Wan D., Yang H., Sanidad KZ., Song M., Bisbee H., Bradbury J., Nan G., Zhang J., Shih P., Lee K., Minter L., Kim D., Xiao H., Liu J., Hammock B., Zeldin D., and Zhang G., Targeted metabolomics identifies cytochrome P450 monooxygenase eicosanoid pathway as novel therapeutic target of colon tumorigenesis. Cancer Research, in press.

4. Nimiya Y*, **Wang W***, Du Z, Sukamtoh E, Zhu J, Decker E, Zhang G: Redox modulation of curcumin stability: Redox active antioxidants increase chemical stability of curcumin. Molecular nutrition & food research 2016, 60(3):487-495. (Featured as front cover of journal)

5. **Wang W***, Sukamtoh E*, Xiao H, Zhang G: Curcumin inhibits lymphangiogenesis in vitro and in vivo. Molecular nutrition & food research 2015, 59(12):2345-2355.

6. Wang W*, Yang J*, Nimiya Y, Lee K, Sanidad KZ, Qi W, Sukamtoh E, Park Y, Liu Z, Zhang G: ω-3 polyunsaturated fatty acids and their cytochrome P450-derived metabolites suppress colorectal tumor development in mice. The Journal of nutritional biochemistry 2017, 48:29-35.

7. **Wang W***, Du Z*, Nimiya Y, Sukamtoh E, Kim D, Zhang G: Allicin inhibits lymphangiogenesis through suppressing activation of vascular endothelial growth factor (VEGF) receptor. The Journal of nutritional biochemistry 2016, 29:83-89.

8. **Wang W***, Yang J*, Qi W, Yang H, Wang C, Tan B, Hammock BD, Park Y, Kim D, Zhang G: Lipidomic profiling of high-fat diet-induced obesity in mice: Importance of

cytochrome P450-derived fatty acid epoxides. Obesity (Silver Spring, Md) 2017, 25(1):132-140

9. **Wang W***, Yang H*, Johnson D, Gensler C, Decker E, Zhang G: Chemistry and biology of omega-3 PUFA peroxidation-derived compounds. Prostaglandins & other lipid mediators 2017,132:84-92.

10. **Wang W***, Yang J*, Yang H, Sanidad KZ, Hammock BD, Kim D, Zhang G: Effects of high-fat diet on plasma profiles of eicosanoid metabolites in mice. Prostaglandins & other lipid mediators 2016, 127:9-13.

11. **Wang W**, Zhu J, Lyu F, Panigrahy D, Ferrara KW, Hammock B, Zhang G: ω-3 polyunsaturated fatty acids-derived lipid metabolites on angiogenesis, inflammation and cancer. Prostaglandins & other lipid mediators 2014, 113-115:13-20.

12. Xu, J., Zhou, L., Ji, L., Chen, F., Fortmann, K., Zhang, K., Liu, Q., Li, K., **Wang W**, Wang, H., Xie, W., Wang, Q., Liu, J., Zheng, B., Zhang, P., Huang, S., Shi, T., Zhang, B., Dang, Y., Chen, J., O'Malley, B. W., Moses, R. E., Wang, P., Li, L., Xiao, J., Hoffmann, A., and Li, X. The REGγ-proteasome forms a regulatory circuit with IκBε and NFκB in experimental colitis, 2016, Nature communications 7, 10761.

13. Wang, Q., Gao, X., Yu, T., Yuan, L., Dai, J., **Wang W.**, Chen, G., Jiao, C., Zhou, W., Huang, Q., Cui, L., Zhang, P., Moses, R. E., Yang, J., Chen, F., Fu, J., Xiao, J., Li, L., Dang, Y.and Li, X., REGγ controls Hippo signaling and reciprocal NF-κB-YAP regulation to promote colon cancer, 2018, Clinical Cancer Research. 2018, doi: 10.1158/1078-0432.CCR-17-2986

14. Wang Y., **Wang W.**, Sanidad K., Shih P., Zhao X., and Zhang G. Eicosanoid signaling in carcinogenesis of colorectal cancer. Cancer Metastasis Review. 2018, doi: 10.1007/s10555-018-9739-8.

15.Sanidad K.*, Yang H.*, **Wang W.**, Ozay E., Yang J., Gu M., Karner M., Zhang J., Kim D., Minter L., Xiao H., and Zhang G. Effects of consumer antimicrobials benzalkonium chloride, benzethonium chloride, and chloroxylenol on colonic inflammation and colitis-associated colon tumorigenesis in mice. Toxicological Science 2018, 163(2): 490-499.

16. Sanidad KZ*, Sukamtoh E*, **Wang W**, Du Z, Florio E, He L, Xiao H, Decker EA, Zhang G: Oxidative Conversion Mediates Antiproliferative Effects of tert-Butylhydroquinone: Structure and Activity Relationship Study. Journal of agricultural and food chemistry 2016, 64(19):3743-3748.

17. Sanidad KZ*, Zhu J*, **Wang W**, Du Z, Zhang G: Effects of Stable Degradation Products of Curcumin on Cancer Cell Proliferation and Inflammation. Journal of agricultural and food chemistry 2016, 64(48):9189-9195.

18. Yang H.*, Du Z.*, **Wang W**, Sukamtoh E., Zheng J., Sanidad K., and Zhang G. Structure and activity relationship of curucmin: role of methoxy groups in its anti-inflammatory and anti-colitis effects. Journal of agricultural and food chemistry. 2017,65: 4509–4515.

19. Yuan B., Ma N., Zhao L., Zhao E., Gao Z., **Wang W**, Song M., Zhang G., Hu Q., Xiao H. In vitro and in vivo inhibitory effects of a Pleurotus eryngii protein on colon cancer cells. Food & Function 2017, 8:3553-3562

20. Yang H., **Wang W.**, and Zhang G. Consumer antimicrobials on gut microbiota and gut health. DNA and Cell Biology, doi: 10.1089/dna.2018.4483.

* co-first author

APPENDIX B

TABLE OF ABBREVIATION

Abbreviation	Full Name
CYP	cytochrome P450
sEH	soluble epoxide hydrolase
PUFAs	polyunsaturated fatty acids
EpFA	epoxygenated fatty acids
LMs	lipid metabolites
COX	cyclooxygenase
LOX	lipoxygenase
LA	α-linolenic acid
γ-LA	γ-linolenic acid
DGLA	dihomo-γ-linolenic acid
EPA	eicosapentaenoic acid
DHA	docosahexaenoic acid
HFD	high-fat diet
LTB4	leukotriene B4
PGI2	prostacyclin
EETs	epoxyeicosatrienoic acids
PTGIS	prostacyclin synthase
PTGES	prostaglandin E synthase
AOM	azoxymethane
DSS	dextran sulfate sodium

BIBLIOGRAPHY

1. Funk, C.D. Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science (New York, N.Y.)* **294**, 1871-1875 (2001).
2. Zeldin, D.C. Epoxygenase pathways of arachidonic acid metabolism. *The Journal of biological chemistry* **276**, 36059-36062 (2001).
3. Zhang, G., Kodani, S. & Hammock, B.D. Stabilized epoxygenated fatty acids regulate inflammation, pain, angiogenesis and cancer. *Prog Lipid Res* **53**, 108-123 (2014).
4. Arnold, C.*, et al.* Arachidonic acid-metabolizing cytochrome P450 enzymes are targets of omega-3 fatty acids. *The Journal of biological chemistry* **285**, 32720-32733 (2010).
5. Wang, W.*, et al.* omega-3 polyunsaturated fatty acids-derived lipid metabolites on angiogenesis, inflammation and cancer. *Prostaglandins Other Lipid Mediat* **113-115**, 13-20 (2014).
6. Siegel, R.L., Miller, K.D. & Jemal, A. Cancer statistics, 2018. *CA Cancer J Clin* **68**, 7-30 (2018).
7. Flegal, K.M., Carroll, M.D., Kit, B.K. & Ogden, C.L. Prevalence of obesity and trends in the distribution of body mass index among US adults, 1999-2010. *JAMA* **307**, 491-497 (2012).
8. Ogden, C.L., Carroll, M.D., Kit, B.K. & Flegal, K.M. Prevalence of obesity and trends in body mass index among US children and adolescents, 1999-2010. *JAMA* **307**, 483-490 (2012).
9. Gulhane, M.*, et al.* High Fat Diets Induce Colonic Epithelial Cell Stress and Inflammation that is Reversed by IL-22. *Scientific reports* **6**, 28990 (2016).
10. Kim, K.-A., Gu, W., Lee, I.-A., Joh, E.-H. & Kim, D.-H. High Fat Diet-Induced Gut Microbiota Exacerbates Inflammation and Obesity in Mice via the TLR4 Signaling Pathway. *PloS one* **7**, e47713 (2012).
11. Liu, Z.*, et al.* Diet-induced obesity elevates colonic TNF-alpha in mice and is accompanied by an activation of Wnt signaling: a mechanism for obesity-associated colorectal cancer. *J Nutr Biochem* **23**, 1207-1213 (2012).
12. Terzić, J., Grivennikov, S., Karin, E. & Karin, M. Inflammation and Colon Cancer. *Gastroenterology* **138**, 2101-2114.e2105 (2010).
13. Moghaddam, A.A., Woodward, M. & Huxley, R. Obesity and risk of colorectal cancer: a meta-analysis of 31 studies with 70,000 events. *Cancer Epidemiol. Biomarkers Prev.* **16**, 2533-2547 (2007).
14. Roberts, D.L., Dive, C. & Renehan, A.G. Biological mechanisms linking obesity and cancer risk: new perspectives. *Annu Rev Med* **61**, 301-316 (2010).
15. Iyer, A., Fairlie, D.P., Prins, J.B., Hammock, B.D. & Brown, L. Inflammatory lipid mediators in adipocyte function and obesity. *Nat Rev Endocrinol* **6**, 71-82 (2010).
16. Zha, W.*, et al.* Functional characterization of cytochrome P450-derived epoxyeicosatrienoic acids in adipogenesis and obesity. *J Lipid Res* **55**, 2124-2136 (2014).
17. Terzic, J., Grivennikov, S., Karin, E. & Karin, M. Inflammation and colon cancer. *Gastroenterology* **138**, 2101-2114 e2105 (2010).

18. Greene, E.R., Huang, S., Serhan, C.N. & Panigrahy, D. Regulation of inflammation in cancer by eicosanoids. *Prostaglandins & other lipid mediators* **96**, 27-36 (2011).
19. Wang, D. & Dubois, R.N. Eicosanoids and cancer. *Nat Rev Cancer* **10**, 181-193 (2010).
20. Chulada, P.C.*, et al.* Genetic disruption of Ptgs-1, as well as Ptgs-2, reduces intestinal tumorigenesis in Min mice. *Cancer Res* **60**, 4705-4708 (2000).
21. Ishikawa, T.O. & Herschman, H.R. Tumor formation in a mouse model of colitis-associated colon cancer does not require COX-1 or COX-2 expression. *Carcinogenesis* **31**, 729-736 (2010).
22. Grosser, T., Fries, S. & FitzGerald, G.A. Biological basis for the cardiovascular consequences of COX-2 inhibition: therapeutic challenges and opportunities. *J Clin Invest* **116**, 4-15 (2006).
23. Wang, Y.*, et al.* Eicosanoid signaling in carcinogenesis of colorectal cancer. *Cancer metastasis reviews* (2018).
24. Lazaar, A.L.*, et al.* Pharmacokinetics, pharmacodynamics and adverse event profile of GSK2256294, a novel soluble epoxide hydrolase inhibitor. *Br J Clin Pharmacol* **81**, 971-979 (2016).
25. McReynolds, C., Schmidt, W.K., Wagner, K. & Hammock, B.D. Advancing Soluble Epoxide Hydrolase Inhibitors Through the Valley of Death into Phase 1 Clinical Trials for Treating Painful Diabetic Neuropathy by Utilizing University Partnerships, Collaborations, and NIH Support. *The FASEB Journal* **30**, 1272.1276 (2016).
26. Veith, H.*, et al.* Comprehensive characterization of cytochrome P450 isozyme selectivity across chemical libraries. *Nature Biotechnology* **27**, 1050 (2009).
27. Yanai, R.*, et al.* Cytochrome P450-generated metabolites derived from omega-3 fatty acids attenuate neovascularization. *Proc Natl Acad Sci U S A* **111**, 9603-9608 (2014).
28. Panigrahy, D.*, et al.* Epoxyeicosanoids stimulate multiorgan metastasis and tumor dormancy escape in mice. *J Clin Invest* **122**, 178-191 (2012).
29. Jiang, J.G.*, et al.* Cytochrome P450 2J2 promotes the neoplastic phenotype of carcinoma cells and is up-regulated in human tumors. *Cancer Res* **65**, 4707-4715 (2005).
30. Pozzi, A.*, et al.* The anti-tumorigenic properties of peroxisomal proliferator-activated receptor alpha are arachidonic acid epoxygenase-mediated. *J Biol Chem* **285**, 12840-12850 (2010).
31. Zhang, W.*, et al.* Soluble epoxide hydrolase deficiency inhibits dextran sulfate sodium-induced colitis and carcinogenesis in mice. *Anticancer Res* **33**, 5261-5271 (2013).
32. Zhang, W.*, et al.* Soluble epoxide hydrolase gene deficiency or inhibition attenuates chronic active inflammatory bowel disease in IL-10(-/-) mice. *Dig Dis Sci* **57**, 2580-2591 (2012).
33. Zhang, W.*, et al.* Reduction of inflammatory bowel disease-induced tumor development in IL-10 knockout mice with soluble epoxide hydrolase gene deficiency. *Mol Carcinog* **52**, 726-738 (2013).

34. Wang, W.*, et al.* Targeted metabolomics identifies cytochrome P450 monooxygenase eicosanoid pathway as novel therapeutic target of colon tumorigenesis. *Cancer Research, in press.*
35. Enayetallah, A.E., French, R.A. & Grant, D.F. Distribution of soluble epoxide hydrolase, cytochrome P450 2C8, 2C9 and 2J2 in human malignant neoplasms. *J Mol Histol* **37**, 133-141 (2006).
36. Willenberg, I.*, et al.* Effect of acute and chronic DSS induced colitis on plasma eicosanoid and oxylipin levels in the rat. *Prostaglandins Other Lipid Mediat* **120**, 155-160 (2015).
37. Vaupel, P. & Mayer, A. Hypoxia in cancer: significance and impact on clinical outcome. *Cancer Metastasis Rev* **26**, 225-239 (2007).
38. Liu, Y.*, et al.* Inhibition of soluble epoxide hydrolase attenuates high-fat-diet-induced hepatic steatosis by reduced systemic inflammatory status in mice. *PloS one* **7**, e39165 (2012).
39. Lopez-Vicario, C.*, et al.* Inhibition of soluble epoxide hydrolase modulates inflammation and autophagy in obese adipose tissue and liver: role for omega-3 epoxides. *Proc Natl Acad Sci U S A* **112**, 536-541 (2015).
40. Wu, C.H.*, et al.* Genetic deletion or pharmacological inhibition of soluble epoxide hydrolase reduces brain damage and attenuates neuroinflammation after intracerebral hemorrhage. *J Neuroinflammation* **14**, 230 (2017).
41. Hanaki, Y.*, et al.* Leukotoxin, 9, 10-epoxy-12-octadecenoate: a possible responsible factor in circulatory shock and disseminated intravascular coagulation. *Jpn J Med* **30**, 224-228 (1991).
42. Hayakawa, M.*, et al.* Proposal of leukotoxin, 9,10-epoxy-12-octadecenoate, as a burn toxin. *Biochem Int* **21**, 573-579 (1990).
43. Hu, J.N.*, et al.* Neutrophil-derived epoxide, 9,10-epoxy-12-octadecenoate, induces pulmonary edema. *Lung* **166**, 327-337 (1988).
44. Kosaka, K., Suzuki, K., Hayakawa, M., Sugiyama, S. & Ozawa, T. Leukotoxin, a linoleate epoxide: its implication in the late death of patients with extensive burns. *Mol Cell Biochem* **139**, 141-148 (1994).
45. Ozawa, T.*, et al.* Cytotoxic activity of leukotoxin, a neutrophil-derived fatty acid epoxide, on cultured human cells. *Biochem Int* **16**, 369-373 (1988).
46. Totani, Y.*, et al.* Leukotoxin and its diol induce neutrophil chemotaxis through signal transduction different from that of fMLP. *Eur Respir J* **15**, 75-79 (2000).
47. Zheng, J., Plopper, C.G., Lakritz, J., Storms, D.H. & Hammock, B.D. Leukotoxin-diol: a putative toxic mediator involved in acute respiratory distress syndrome. *Am J Respir Cell Mol Biol* **25**, 434-438 (2001).
48. Moghaddam, M.F.*, et al.* Bioactivation of leukotoxins to their toxic diols by epoxide hydrolase. *Nat Med* **3**, 562-566 (1997).
49. Node, K.*, et al.* Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. *Science* **285**, 1276-1279 (1999).
50. Morin, C., Sirois, M., Echave, V., Gomes, M.M. & Rousseau, E. EET displays anti-inflammatory effects in TNF-alpha stimulated human bronchi: putative role of CPI-17. *Am J Respir Cell Mol Biol* **38**, 192-201 (2008).

51. Zhang, G.*, et al.* Epoxy metabolites of docosahexaenoic acid (DHA) inhibit angiogenesis, tumor growth, and metastasis. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 6530-6535 (2013).
52. Wang, W.*, et al.* omega-3 Polyunsaturated fatty acids and their cytochrome P450-derived metabolites suppress colorectal tumor development in mice. *J Nutr Biochem* **48**, 29-35 (2017).
53. Yang, W.*, et al.* Characterization of epoxyeicosatrienoic acid binding site in U937 membranes using a novel radiolabeled agonist, 20-125i-14,15-epoxyeicosa-8(Z)-enoic acid. *J Pharmacol Exp Ther* **324**, 1019-1027 (2008).
54. Chen, Y., Falck, J.R., Manthati, V.L., Jat, J.L. & Campbell, W.B. 20-Iodo-14,15-epoxyeicosa-8(Z)-enoyl-3-azidophenylsulfonamide: Photoaffinity Labeling of a 14,15-Epoxyeicosatrienoic Acid Receptor. *Biochemistry* **50**, 3840-3848 (2011).
55. Park, S.K.*, et al.* GPR40 is a low-affinity epoxyeicosatrienoic acid receptor in vascular cells. *J Biol Chem* **293**, 10675-10691 (2018).
56. Liu, X.*, et al.* Functional screening for G protein-coupled receptor targets of 14,15-epoxyeicosatrienoic acid. *Prostaglandins Other Lipid Mediat* **132**, 31-40 (2017).
57. Li, P.*, et al.* Epoxyeicosatrienoic acids enhance embryonic haematopoiesis and adult marrow engraftment. *Nature* **523**, 468-471 (2015).
58. Ding, Y.*, et al.* The biological actions of 11,12-epoxyeicosatrienoic acid in endothelial cells are specific to the R/S-enantiomer and require the G(s) protein. *J Pharmacol Exp Ther* **350**, 14-21 (2014).
59. Kundu, S.*, et al.* Metabolic products of soluble epoxide hydrolase are essential for monocyte chemotaxis to MCP-1 in vitro and in vivo. *J Lipid Res* **54**, 436-447 (2013).
60. Hu, J.*, et al.* Muller glia cells regulate Notch signaling and retinal angiogenesis via the generation of 19,20-dihydroxydocosapentaenoic acid. *J Exp Med* **211**, 281-295 (2014).
61. Hu, J.*, et al.* Inhibition of soluble epoxide hydrolase prevents diabetic retinopathy. *Nature* **552**, 248-252 (2017).
62. Enos, R.T.*, et al.* High-fat diets rich in saturated fat protect against azoxymethane/dextran sulfate sodium-induced colon cancer. *Am J Physiol Gastrointest Liver Physiol* **310**, G906-919 (2016).
63. Wu, B.*, et al.* Dietary corn oil promotes colon cancer by inhibiting mitochondria-dependent apoptosis in azoxymethane-treated rats. *Experimental biology and medicine (Maywood, N.J.)* **229**, 1017-1025 (2004).
64. Fujise, T.*, et al.* Long-term feeding of various fat diets modulates azoxymethane-induced colon carcinogenesis through Wnt/beta-catenin signaling in rats. *Am J Physiol Gastrointest Liver Physiol* **292**, G1150-1156 (2007).
65. Reddy, B.S., Tanaka, T. & Simi, B. Effect of different levels of dietary trans fat or corn oil on azoxymethane-induced colon carcinogenesis in F344 rats. *J Natl Cancer Inst* **75**, 791-798 (1985).
66. Pot, G.K.*, et al.* Opposing associations of serum n-3 and n-6 polyunsaturated fatty acids with colorectal adenoma risk: an endoscopy-based case-control study. *Int J Cancer* **123**, 1974-1977 (2008).

67. Ogden, C.L., Carroll, M.D., Fryar, C.D. & Flegal, K.M. Prevalence of Obesity Among Adults and Youth: United States, 2011-2014. *NCHS Data Brief*, 1-8 (2015).
68. Ma, Y.*, et al.* Obesity and Risk of Colorectal Cancer: A Systematic Review of Prospective Studies. *PLoS ONE* **8**, e53916 (2013).
69. Wang, W.*, et al.* Lipidomic profiling reveals soluble epoxide hydrolase as a therapeutic target of obesity-induced colonic inflammation. *Proc Natl Acad Sci U S A* **115**, 5283-5288 (2018).
70. Bettaieb, A.*, et al.* Soluble epoxide hydrolase deficiency or inhibition attenuates diet-induced endoplasmic reticulum stress in liver and adipose tissue. *J Biol Chem* **288**, 14189-14199 (2013).
71. De Taeye, B.M.*, et al.* Expression and regulation of soluble epoxide hydrolase in adipose tissue. *Obesity (Silver Spring)* **18**, 489-498 (2010).
72. do Carmo, J.M.*, et al.* Inhibition of soluble epoxide hydrolase reduces food intake and increases metabolic rate in obese mice. *Nutr Metab Cardiovasc Dis* **22**, 598-604 (2012).
73. Imig, J.D.*, et al.* Soluble epoxide hydrolase inhibition and peroxisome proliferator activated receptor gamma agonist improve vascular function and decrease renal injury in hypertensive obese rats. *Experimental biology and medicine (Maywood, N.J.)* **237**, 1402-1412 (2012).
74. Iyer, A.*, et al.* Pharmacological inhibition of soluble epoxide hydrolase ameliorates diet-induced metabolic syndrome in rats. *Experimental diabetes research* **2012**, 758614 (2012).
75. Roche, C.*, et al.* Soluble epoxide hydrolase inhibition improves coronary endothelial function and prevents the development of cardiac alterations in obese insulin-resistant mice. *Am J Physiol Heart Circ Physiol* **308**, H1020-1029 (2015).
76. Zhang, L.N.*, et al.* Inhibition of soluble epoxide hydrolase attenuates endothelial dysfunction in animal models of diabetes, obesity and hypertension. *European journal of pharmacology* **654**, 68-74 (2011).
77. Ogden, C.L., Carroll, M.D., Kit, B.K. & Flegal, K.M. Prevalence of childhood and adult obesity in the United States, 2011-2012. *JAMA : the journal of the American Medical Association* **311**, 806-814 (2014).
78. Must, A.*, et al.* The disease burden associated with overweight and obesity. *JAMA : the journal of the American Medical Association* **282**, 1523-1529 (1999).
79. Spieker, E.A. & Pyzocha, N. Economic Impact of Obesity. *Primary care* **43**, 83-95, viii-ix (2016).
80. Hodnett, B.L., Dearman, J.A., Carter, C.B. & Hester, R.L. Attenuated PGI2 synthesis in obese Zucker rats. *American journal of physiology. Regulatory, integrative and comparative physiology* **296**, R715-721 (2009).
81. Li, P.*, et al.* LTB4 promotes insulin resistance in obese mice by acting on macrophages, hepatocytes and myocytes. *Nature medicine* **21**, 239-247 (2015).
82. Zhou, Y.*, et al.* Gender differences of renal CYP-derived eicosanoid synthesis in rats fed a high-fat diet. *American journal of hypertension* **18**, 530-537 (2005).
83. Theken, K.N.*, et al.* Enalapril reverses high-fat diet-induced alterations in cytochrome P450-mediated eicosanoid metabolism. *American journal of physiology. Endocrinology and metabolism* **302**, E500-509 (2012).

84. Astarita, G., Kendall, A.C., Dennis, E.A. & Nicolaou, A. Targeted lipidomic strategies for oxygenated metabolites of polyunsaturated fatty acids. *Biochimica et biophysica acta* **1851**, 456-468 (2015).
85. Yang, J., Dong, H. & Hammock, B.D. Profiling the regulatory lipids: another systemic way to unveil the biological mystery. *Current opinion in lipidology* **22**, 197-203 (2011).
86. Yang, J., Schmelzer, K., Georgi, K. & Hammock, B.D. Quantitative profiling method for oxylipin metabolome by liquid chromatography electrospray ionization tandem mass spectrometry. *Analytical chemistry* **81**, 8085-8093 (2009).
87. Hariri, N. & Thibault, L. High-fat diet-induced obesity in animal models. *Nutrition research reviews* **23**, 270-299 (2010).
88. Juaneda, P. & Rocquelin, G. Rapid and convenient separation of phospholipids and non phosphorus lipids from rat heart using silica cartridges. *Lipids* **20**, 40-41 (1985).
89. Chen, W.*, et al.* Altered lipid metabolism in residual white adipose tissues of Bscl2 deficient mice. *PloS one* **8**, e82526 (2013).
90. Morisseau, C. & Hammock, B.D. Impact of soluble epoxide hydrolase and epoxyeicosanoids on human health. *Annu Rev Pharmacol Toxicol* **53**, 37-58 (2013).
91. Ye, D.*, et al.* Cytochrome p-450 epoxygenase metabolites of docosahexaenoate potently dilate coronary arterioles by activating large-conductance calcium-activated potassium channels. *J. Pharmacol. Exp. Ther.* **303**, 768-776 (2002).
92. Cheng, Y.*, et al.* Role of prostacyclin in the cardiovascular response to thromboxane A2. *Science (New York, N.Y.)* **296**, 539-541 (2002).
93. Virtue, S.*, et al.* Prostaglandin profiling reveals a role for haematopoietic prostaglandin D synthase in adipose tissue macrophage polarisation in mice and humans. *International journal of obesity (2005)* **39**, 1151-1160 (2015).
94. Neuhofer, A.*, et al.* Impaired local production of proresolving lipid mediators in obesity and 17-HDHA as a potential treatment for obesity-associated inflammation. *Diabetes* **62**, 1945-1956 (2013).
95. Hetu, P.O. & Riendeau, D. Down-regulation of microsomal prostaglandin E2 synthase-1 in adipose tissue by high-fat feeding. *Obesity (Silver Spring, Md.)* **15**, 60-68 (2007).
96. Claria, J., Dalli, J., Yacoubian, S., Gao, F. & Serhan, C.N. Resolvin D1 and resolvin D2 govern local inflammatory tone in obese fat. *Journal of immunology (Baltimore, Md. : 1950)* **189**, 2597-2605 (2012).
97. Lieb, D.C.*, et al.* Adipose tissue 12/15 lipoxygenase pathway in human obesity and diabetes. *The Journal of clinical endocrinology and metabolism* **99**, E1713-1720 (2014).
98. Grosser, T., Fries, S. & FitzGerald, G.A. Biological basis for the cardiovascular consequences of COX-2 inhibition: therapeutic challenges and opportunities. *The Journal of Clinical Investigation* **116**, 4-15 (2006).
99. Johnson, R.L. & Fleet, J.C. Animal models of colorectal cancer. *Cancer metastasis reviews* **32**, 39-61 (2013).

100. Scheer, N., *et al.* Generation and characterization of novel cytochrome P450 Cyp2c gene cluster knockout and CYP2C9 humanized mouse lines. *Molecular pharmacology* **82**, 1022-1029 (2012).
101. Megaraj, V., *et al.* Role of hepatic and intestinal p450 enzymes in the metabolic activation of the colon carcinogen azoxymethane in mice. *Chemical research in toxicology* **27**, 656-662 (2014).
102. Makita, K., *et al.* Experimental and/or genetically controlled alterations of the renal microsomal cytochrome P450 epoxygenase induce hypertension in rats fed a high salt diet. *J Clin Invest* **94**, 2414-2420 (1994).
103. Node, K., *et al.* Anti-inflammatory Properties of Cytochrome P450 Epoxygenase-Derived Eicosanoids. *Science (New York, N.Y.)* **285**, 1276-1279 (1999).
104. Ozawa, T., *et al.* Leukotoxin, 9,10-epoxy-12-octadecenoate, as a burn toxin causing adult respiratory distress syndrome. *Advances in prostaglandin, thromboxane, and leukotriene research* **21B**, 569-572 (1991).
105. Fukushima, A., *et al.* Cardiovascular effects of leukotoxin (9, 10-epoxy-12-octadecenoate) and free fatty acids in dogs. *Cardiovascular research* **22**, 213-218 (1988).
106. Ozawa, T., *et al.* Biosynthesis of leukotoxin, 9,10-epoxy-12 octadecenoate, by leukocytes in lung lavages of rat after exposure to hyperoxia. *Biochem Biophys Res Commun* **134**, 1071-1078 (1986).
107. Blasbalg, T., Hibbeln, J., Ramsden, C., Majchrzak, S. & Rawlings, R. Changes in consumption of omega-3 and omega-6 fatty acids in the United States during the 20th century. *The American journal of clinical nutrition* **93**, 950 - 962 (2011).
108. Siegel, R.L., Miller, K.D. & Jemal, A. Cancer statistics, 2016. *CA: a cancer journal for clinicians* **66**, 7-30 (2016).
109. Sasazuki, S., *et al.* Intake of n-3 and n-6 polyunsaturated fatty acids and development of colorectal cancer by subsite: Japan Public Health Center-based prospective study. *International journal of cancer. Journal international du cancer* **129**, 1718-1729 (2011).
110. Murff, H.J., *et al.* Dietary intake of PUFAs and colorectal polyp risk. *The American journal of clinical nutrition* **95**, 703-712 (2012).
111. Kim, S., Sandler, D.P., Galanko, J., Martin, C. & Sandler, R.S. Intake of polyunsaturated fatty acids and distal large bowel cancer risk in whites and African Americans. *Am J Epidemiol* **171**, 969-979 (2010).
112. Hall, M.N., Chavarro, J.E., Lee, I.M., Willett, W.C. & Ma, J. A 22-year prospective study of fish, n-3 fatty acid intake, and colorectal cancer risk in men. *Cancer Epidemiol. Biomarkers Prev.* **17**, 1136-1143 (2008).
113. Schloss, I., Kidd, M.S., Tichelaar, H.Y., Young, G.O. & O'Keefe, S.J. Dietary factors associated with a low risk of colon cancer in coloured west coast fishermen. *South African medical journal = Suid-Afrikaanse tydskrif vir geneeskunde* **87**, 152-158 (1997).
114. Simopoulos, A.P. The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* **56**, 365-379 (2002).
115. Jump, D.B. The biochemistry of n-3 polyunsaturated fatty acids. *The Journal of biological chemistry* **277**, 8755-8758 (2002).

116. Fischer, R.*, et al.* Dietary Omega-3 Fatty Acids Modulate the Eicosanoid Profile in Man Primarily via the CYP-epoxygenase Pathway. *Journal of lipid research* (2014).
117. Zivkovic, A.*, et al.* Serum oxylipin profiles in IgA nephropathy patients reflect kidney functional alterations. *Metabolomics* **8**, 1102-1113 (2012).
118. Cathcart, M.C., Lysaght, J. & Pidgeon, G.P. Eicosanoid signalling pathways in the development and progression of colorectal cancer: novel approaches for prevention/intervention. *Cancer metastasis reviews* **30**, 363-385 (2011).
119. Simopoulos, A.P., Leaf, A. & Salem, N., Jr. Essentiality of and recommended dietary intakes for omega-6 and omega-3 fatty acids. *Ann Nutr Metab* **43**, 127-130 (1999).
120. Aslan, A. & Triadafilopoulos, G. Fish oil fatty acid supplementation in active ulcerative colitis: a double-blind, placebo-controlled, crossover study. *The American journal of gastroenterology* **87**, 432-437 (1992).
121. Salomon, P., Kornbluth, A.A. & Janowitz, H.D. Treatment of ulcerative colitis with fish oil n--3-omega-fatty acid: an open trial. *Journal of clinical gastroenterology* **12**, 157-161 (1990).
122. Belluzzi, A.*, et al.* Polyunsaturated fatty acids and inflammatory bowel disease. *The American journal of clinical nutrition* **71**, 339S-342S (2000).
123. Akedo, I.*, et al.* Three cases with familial adenomatous polyposis diagnosed as having malignant lesions in the course of a long-term trial using docosahexanoic acid (DHA)-concentrated fish oil capsules. *Japanese journal of clinical oncology* **28**, 762-765 (1998).
124. Kobayashi, M.*, et al.* Fish, long-chain n-3 polyunsaturated fatty acids, and risk of colorectal cancer in middle-aged Japanese: the JPHC study. *Nutrition and cancer* **49**, 32-40 (2004).
125. Stern, M.C.*, et al.* Polyunsaturated fatty acids, DNA repair single nucleotide polymorphisms and colorectal cancer in the Singapore Chinese Health Study. *Journal of nutrigenetics and nutrigenomics* **2**, 273-279 (2009).
126. Woodworth, H.L.*, et al.* Dietary fish oil alters T lymphocyte cell populations and exacerbates disease in a mouse model of inflammatory colitis. *Cancer Res* **70**, 7960-7969 (2010).
127. Srivastava, P.K., Sharma, V.K., Kalonia, D.S. & Grant, D.F. Polymorphisms in human soluble epoxide hydrolase: effects on enzyme activity, enzyme stability, and quaternary structure. *Archives of biochemistry and biophysics* **427**, 164-169 (2004).
128. Dreisbach, A.W.*, et al.* The Prevalence of CYP2C8, 2C9, 2J2, and soluble epoxide hydrolase polymorphisms in African Americans with hypertension. *American journal of hypertension* **18**, 1276-1281 (2005).
129. Spiecker, M.*, et al.* Risk of coronary artery disease associated with polymorphism of the cytochrome P450 epoxygenase CYP2J2. *Circulation* **110**, 2132-2136 (2004).
130. Lee, C.R.*, et al.* Genetic variation in soluble epoxide hydrolase (EPHX2) and risk of coronary heart disease: The Atherosclerosis Risk in Communities (ARIC) study. *Human molecular genetics* **15**, 1640-1649 (2006).

131. Wei, Q.*, et al.* Sequence variation in the soluble epoxide hydrolase gene and subclinical coronary atherosclerosis: interaction with cigarette smoking. *Atherosclerosis* **190**, 26-34 (2007).
132. Fornage, M.*, et al.* Polymorphism of the soluble epoxide hydrolase is associated with coronary artery calcification in African-American subjects: The Coronary Artery Risk Development in Young Adults (CARDIA) study. *Circulation* **109**, 335-339 (2004).
133. Przybyla-Zawislak, B.D.*, et al.* Polymorphisms in human soluble epoxide hydrolase. *Molecular pharmacology* **64**, 482-490 (2003).
134. Simopoulos, A.P. Genetic variants in the metabolism of omega-6 and omega-3 fatty acids: their role in the determination of nutritional requirements and chronic disease risk. *Experimental biology and medicine (Maywood, N.J.)* **235**, 785-795 (2010).
135. Zeisel, S.H.*, et al.* Highlights of the 2012 Research Workshop: Using nutrigenomics and metabolomics in clinical nutrition research. *JPEN. Journal of parenteral and enteral nutrition* **37**, 190-200 (2013).